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Production of HIV-2 Envelope Glycoproteins for Structural and Functional Studies

A Thesis submitted to the University of London for the Degree of Doctor of Philosophy

Jennifer Halley

Division of Virology
The National Institute for Medical Research
The Ridgeway
Mill Hill
London
NW7 1AA

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Envelope glycoproteins of HIV-1 (gp160⇒gp120/41) and HIV-2 (gp140⇒gp105/36) have ~40% sequence identity, bind cell surface receptors (CD4 and CCR5 or CXCR4) and effect membrane fusion, suggesting a close structural similarity. HIV-2 gp140 is more stable than HIV-1 gp160 (Sattentau et al., 1993), possibly making it a better candidate for structural studies. We have generated HIV-2 *env*-gene constructs that allow expression of soluble gp120 (gp105 with a truncated gp36 [gp15] lacking the membrane anchor and cytoplasmic tail) in mammalian cells.

HIV-2 *env*-genes have been rescued from the HIV-2_{ROD} prototype and six HIV-2-infected individuals resident in Caio, Guinea Bissau. A range of constructs have been generated to enable efficient secretion of gp120s into tissue culture supernatant (TCSN) and/or enhance stability of the glycoproteins. These result in truncation at one of two positions in the gp36 coding region upstream of the membrane anchor, removal of the gp105/36 processing site and, introduction of a trimer-stabilising motif from Bacteriophage T4 fibritin (GYIPEAPRDGQAYVRKDG-EWVLLSTFL) at the C-terminus of gp15. All constructs were cloned to express a hexa-His tag at their C-termini to aid purification of the protein, but this proved inefficient.

All constructs have been screened for expression competence in a transient system based on transfection of Human Embryonic Kidney 293T cells and western blot detection of gp120 in cell lysates and TCSN. Seven HIV-2_{ROD} stable cell lines, based on Chinese Hamster Ovary Cells (CHO K1), have been selected which constitutively express and secrete gp120. The secreted material is recognised by a panel of mapped anti-gp105 monoclonal antibodies (MAbs) and partially purified gp120 is functionally active for human CD4-binding in ELISA-based assays, indicating that it is probably in a native state. Purification is based on lectin (GNA)-affinity chromatography followed by a MAb immunoaffinity column (ARP 3085, NIBSC) and gel filtration (Superose 6). This, together with dynamic light scattering (polydispersity ~23.3) and circular dichroism (performed up to 85°C) suggests

production of a correctly folded, oligomeric (probably trimeric) protein of a purity suitable for structural studies. Purified proteins are being used to set up further structural and functional experiments including crystallisation trials and will be used to generate MAbs which may assist crystallisation and be useful for HIV-2 strain typing assays.

Declaration

I, Jennifer Halley, declare that the work described in this thesis was, except where otherwise indicated in the text, entirely my own.

I have not submitted any portion of the work referred to in this thesis in support of any other qualification at this or any other institute of higher learning.

Jennifer Halley, April 2007

To Mum, Dad, Sis
and Vasantha

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Thank you all

Glossary Of Terms

293T	Human Embryonic Kidney cell line transformed with the Simian Virus 40 large T antigen
AH1/2	Amphipathic Helix 1/2
AIDS	Acquired Immunodeficiency Syndrome
AMD3100	CXCR4 Inhibitor
Amp	Ampicillin
ANC/Anc	Membrane anchor
APOBEC	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like
ARC	AIDS related complex
AUC	Analytical Ultracentrifugation
Ass	Assembly Domain
BCA	B-cell activating chemokine
BIV	Bovine Immunodeficiency Virus
BLC	B lymphocyte chemoattractant
BSA	Bovine Serum Albumin
CAEV	Caprine Arthritis Encephalitis Virus
CAF	CD8 antiviral factors
Cat. No.	Catalogue Number
CC	or β-chemokines with two adjacent cysteines near the amino terminus of the protein
CCL3/4/5	β chemokines
CCR2/5	β chemokine receptors
CD	Circular Dichroism
(s)CD4	(soluble) Cell surface marker expressed by a subset of immune cells
CD8	Cell surface marker expressed by a subset of immune cells
CDC	Center for Disease Control
CFAR	Centralised facility for AIDS reagents
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO K1	Chinese Hamster Ovary cell line
CMV	Cytomegalovirus
CNS	Central Nervous System

cs	Cleavage site mutant
CSW	Commercial Sex Workers
CTL	Cytolytic T Lymphocytes
CV-1	African green monkey kidney fibroblast cell line
CVS	Cervico-vaginal secretions
CXC	or α-chemokines in which the cysteines at the amino terminus of the protein are separated by an amino acid
CXCR4	β chemokine receptor
DAPI	4'6-Diamidino-2-phenylindole dihydrochloride
DC	dendritic cells
DC-SIGN	Dendritic cell–specific ICAM-3-grabbing nonintegrin
DLS	Dynamic Light Scattering
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulfoxide
(ds)DNA	(double stranded) Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DRC	Democratic Republic of the Congo
DTT	Dithiothreitol
EC_{50}	represents the concentration of a compound where 50% of its effect is observed
ECL	Enhanced-chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EIAV	Equine Infectious Anemia Virus
ELISA	Enzyme-linked immunosorbent assay
env	HIV gene encoding Env
Fab	Antibody fragment
Fas	is the most intensly studied protein of the apoptotic receptor pathway
FCS	Foetal calf serum
FIB	Fibritin domain
FIV	Feline Immunodeficiency Virus
FP	Fusion peptide
FPLC	Fast protein liquid chromatography
FTIR	Fourier transform infrared spectroscopy

Gag	or p55 Gag polyprotein precursor
GCP	Granulocyte chemoattractant protein
GDE	Genetic data environment
GlcNAc	N-Acetylglucosaminyl
gp120	Soluble ectodomain of HIV-2 envelope glycoprotein
gp140	HIV-2 precursor envelope glycoprotein. Also the soluble ectodomain of HIV-1 envelope glycoprotein
gp160	HIV-1 precursor envelope glycoprotein
gp41/gp36	HIV-1/HIV-2 transmembrane domain
GPCR	G-protein coupled receptor
GRO	Growth-related oncogene
GS	Glutamine Synthetase
HA	Influenza haemagglutinin
hA3G	human apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like (APOBEC) 3 G
HAART	Highly active anti-retroviral therapy
HBS	HEPES-buffered saline
HCMV	human cytomegalovirus
HLA	Human Leukocyte Antigen
HIS-tag	hexa-His Tag
HIV	Human Immunodeficiency Virus
HR1/2	Helical regions ½ of the ectodomain of gp41/36
HRP	Horse radish peroxidase
HT	Sodium hypoxanthine and thymidine supplement
HTLV	Human T-lymphotropic Virus
IDU	Intravenous Drug Users
IEF	Isoelectric Focusing
IEX	Ion Exchange Chromatography
IFC	Integrated µ-Fluidic Cartridge
Ig	Immunoglobulin
IL	Interleukin
IN	Integrase
INF	Interferon

IP	Immunoprecipitation
IPG	Immobilised pH gradient buffer
K_d	Binding Affinity
Ken	Kennedy Domain
LAV	Lymphadenopathy AIDS Virus
LTR	Long Terminal Repeat
LZL	Leucine-Zipper-Like domain
MA	Matrix
MAb	Monoclonal Antibody
MALT	Mucosal-associated lymphoid tissue
MCP	Monocyte chemotactic protein
MDC	Macrophage derived chemokine
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Proteins
MRW	Mean residue weight
MSX	L-Methionine Sulphoximine
MuLV	Murine Leukaemia Virus
MVV	Maedi/Visna Virus
MW	Molecular Weight
Naf	Nafcillin
NEB	New England Biolabs
Nef	Negative factor
NFκB	Nuclear Factor kappa beta
NIBSC	National Institute for Biological Standards and Controls
NIH	National Institutes of Health
NK	Natural Killer Cells
NLS	Nuclear Localisation Signal (NLS)
NMR	Nuclear Magnetic Resonance
(N)NRTI	(Non)Nucleoside reverse transcriptase inhibitors
NTPs	Nucleoside triphosphates
OIV	Ovine Immunodeficiency Virus
$P56^{lck}$	Protein tyrosine kinase
PAGE	Polyacrylamide gel electrophoresis

PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PBT	Phosphate buffered saline containing 0.01% of Tween 20
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PDC	Plasmacytoid dendritic cells
PDI	Protein disulphide isomerase
PI	Protease Inhibitor
PIC	Pre-integration complex
Pol	Pol polyprotein precursor
Pol II	RNA essential to the transcriptional apparatus
P/S	Penicillin/Streptomycin
RANTES	Regulated upon activation normal T-cells expressed and secreted
Rev	Regulator of viral protein expression
RIPA	RadioImmunoPrecipitation Assay
(m/t/v)RNA	(messenger/transfer/viral) Ribonucleic acid
RRE	Rev-response element
RPMI	Roswell Park Memorial Institute Medium
RT	Reverse Transcriptase
SAC	Protein A Staphlycoccus aureus
SB 3-10	3-(Decyldimethylammino)propanesulfonate
SDF-1	Stromal derived factor 1 (ligand of CXCR4)
SDS	Sodium dodecyl sulphate
Sig	Signal Peptide
SHIV	Simian-human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SOE	Splice Overlap Extension
SP	Signal peptide
Sp1	transactivator protein
SPR BIAcore	Surface Plasmon resonance BIAcore
STD	Sexually Transmitted Diseases
STLV	Simian T-lymphotropic virus
SU	Surface glycoprotein. Also referred to as gp105 (HIV-2)/gp120

	(HIV-1)
T-20/T-1249	Fusion Inhibitors
TAE	Tris-acetate EDTA
TAR	Tat-responsive element
TARC	Thymus and activation related chemokine
Tat	transactivating protein
TB	Tuberculosis
TBE	Tris-borate EDTA
TCR	T-cell receptor
TCSN	Tissue Culture Supernatant
TECK	Thymus-expressed chemokine
Th1/Th2	T/B cell dominated immune response
TM	Transmembrane glycoprotein. Also referred to as gp36 (HIV-2)/gp41 (HIV-1)
TNF	Tumour Necrosis Factor
tPA SP	Tissue plasminogen activator signal peptide
Tris	Tris(hydroxymethyl)methylamine
UK	United Kingdom
UNAIDS	Joint United Nations programme on HIV and AIDS
USA	United States of America
UV	Ultra-violet
Vif	viral infectivity factor
VLP	Virus Like Particle
Vpr	viral protein r
Vpu	viral protein u
Vpx	viral protein x
WHO	World Health Organisation

Amino Acid Nomenclature

Standard one and three letter amino acid codes are used throughout the text. The table below describes the three and single letter amino acid designations.

Amino Acid	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Iso	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1

Introduction

1 Introduction

The introduction into the human population of a virus, associated with immunodeficiency, from non-human primates was not a unique event. It is estimated that Human Immunodeficiency Virus Type 1 (HIV-1) established itself in the human population in 1931 (95% confidence interval 1915-1941) and some ten years later Human Immunodeficiency Virus Type 2 (HIV-2) made the species jump from sooty mangabeys (*Cercocebus torquatus atys*) (Korber et al., 2000; Lemey et al., 2003). Both HIV-1 and HIV-2 have been assigned to the 'primate Lentivirus' subgenus in the *Retroviridae* family and as the name Lentivirus suggests there is a slow progression to disease (Ratner et al., 1985; Wain-Hobson et al., 1985). Infection with either virus attacks the patients' internal defence mechanisms eventually causing progression to Acquired ImmunoDeficiency Syndrome (AIDS) (Clavel et al., 1986b; Gottlieb et al., 1981b). Current reports from UNAIDS estimate that there are 40 million people around the world infected with HIV and there are approximately 5 million new infections per year (WHO, 2005). Despite the numerous anti-retroviral drugs on the market we are currently unable to either completely clear HIV from patients or prevent infection with a vaccine or microbicide. The virus continually adapts to overcome the drugs, hence we can suppress the virus for a limited period of time only, after which the patients succumb to the disease.

1.1 Aetiology of AIDS

Twenty five years ago clinicians in California and New York noticed cases of rare diseases, generally associated with immunosuppressed patients, in homosexual men (Friedman-Kien et al., 1981; Gottlieb et al., 1981a). Following these reports a paper published in the New England Journal of Medicine showed that these patients had a common immunological deficit in cell mediated immunity due to a severe loss of circulating $CD4^+$ T cells (Gottlieb et al., 1981b; Masur et al., 1981). It became clear that this AIDS was caused by an as yet unidentified infectious agent transferred from person to person by intimate contact or through blood (Francis, Curran, and Essex, 1983).

This coincided with the discovery of the first human retrovirus, human T lymphotropic virus I (HTLV-I) which was known to infect T cells (Poiesz et al., 1980). Therefore, it seemed possible that the aetiological agent of AIDS could be an undiscovered retrovirus. Two groups, one at the Pasteur Institute in Paris headed by Luc Montagnier and the other at the National Institute for Health in the U.S.A. led by Robert Gallo discovered the cause of AIDS called either lymphadenopathy-associated virus (LAV) or human T lymphotropic virus III (HTLV-III) respectively (Barre-Sinoussi et al., 1983; Gallo et al., 1983). The virus was also isolated independently by Jay Levy in California from both individuals showing symptoms of AIDS and asymptomatic individuals from groups at high risk for AIDS (Levy et al., 1984). The virus isolated by all these groups was later called Human Immunodeficiency Virus Type 1 (HIV-1) (Gallo and Montagnier, 1987).

In 1985, a retrovirus called simian T-cell lymphotropic virus type III (STLV-III) or Simian Immunodeficiency Virus (SIV) was isolated in the U.S.A. from captive macaques displaying AIDS-like disease (Daniel et al., 1985). The morphology, growth characteristics and antigenic properties of this virus were found to be very similar to those of HIV-1 and macaques were suggested as a possible animal model (Daniel et al., 1985). Later studies have found that SIV infects at least 36 non-human primate species in sub-Saharan Africa (Bibollet-Ruche et al., 2004; Peeters et al., 2002), including chimpanzees (*Pan troglodytes troglodytes*) and the sooty mangabey (*Cercocebus t. atys*) (Franchini et al., 1987; Hirsch et al., 1989; Marx et al., 1991).

In July of 1986 Clavel and colleagues (Clavel et al., 1986a; Clavel et al., 1986b) reported the isolation of a new human retrovirus from West African patients with AIDS. Two male patients, from Guinea Bissau and Cape Verde, were negative for HIV-1 antibodies by ELISA, Western Blot and radioimmunoprecipitation assay but they both showed symptoms of AIDS according to the U.S.A. Center for Disease Control (CDC) criteria (Table 1.1). Electron microscopy examination of their infected cells revealed the presence of mature virions and budding of viral particles similar to those of HIV-1. This new virus showed greater serological cross reactivity with (Clavel et al., 1986c), and was later confirmed to have 75% sequence

homology with, SIV from infected macaques (Chakrabarti et al., 1987; Guyader et al., 1987; Hirsch et al., 1989). It was named lymphadenopathy-associated virus II (LAV II) and later called Human Immunodeficiency Virus Type 2 (HIV-2).

	Clinical Categories		
	A	B	C
CD4 cell categories	Asymptomatic, lymphadenopathy, or acute infection	Symptomatic,[a] not category A or C	Clinical AIDS[b]
>500/μL (>29%)	A1	B1	C1
200-499/μL (14-28%)	A2	B2	C2
<200/μL (<14%)	A3	B3	C3

Table 1.1: AIDS surveillance case definition for adults and adolescents

Categories A3, B3 and C1-3 are defined as AIDS

[a]Examples of these symptoms include bacillary angiomatosis; thrush; vulvovaginal candidiasis that is persistent, frequent, or poorly responsive to therapy; cervical dysplasia or carcinoma in situ; constitutional symptoms such as fever or diarrhoea of > 1month duration; oral hairy leukoplakia; multidermatomal or recurrent herpes zoster; immune thrombocytopenic purpura; listeriosis; pelvic inflammatory disease; and peripheral neuropathy.

[b]Candidiasis of the oesophagus or respiratory tract; invasive cervical cancer; extra pulmonary coccidioidomycosis; extra pulmonary cryptococcosis; cryptosporidiosis; extralymphatic cytomegalovirus infection; herpes simplex with mucocutaneous ulcer > 1 month duration, or bronchitis, pneumonitis, or oesophagitis; extrapulmonary histoplasmosis; HIV-associated dementia; wasting syndrome; isosporiasis; Karposi's sarcoma; CNS lymphoma; non-Hodgkin's lymphoma; pulmonary tuberculosis; disseminated *Mycobacteria tuberculosis*, *M. avium*, or *M. kansasii* infection; nocardiosis; *Pneumocystis carinii* pneumonia; recurrent bacterial pneumonia; progressive multi-focal leukoencephalopathy; recurrent *Salmonella* septicaemia; extraintestinal strongyloidiasis; toxoplasmosis. Adapted from (Castro et al., 1992).

1.2 Retroviruses

The *Retroviridae* family is a large and diverse group of viruses which are found in all vertebrates. The unique nature of their lifecycle, reverse transcribing their genomic RNA into DNA that can then integrate into the host chromosome, distinguishes this family of viruses. The genera were originally defined by the morphology of the core in the mature virus. New criteria are used now involving the complexity of the virus and sequence homology. The International Committee on Taxonomy of Viruses formalised the genera by splitting the *Retroviridae* into two main groups the "simple" and the "complex" retroviruses. The "simple" viruses encode only the Gag, Pol, and Env gene products and these are the alpha, beta and gammaretroviruses. The "complex" viruses encode these gene products and also several small regulatory proteins with a variety of functions. The deltaretroviruses, epsilonretroviruses, lentiviruses and spumaviruses are considered "complex".

The particle associated reverse transcriptase activity was one of the first characteristics of HIV-1 that members of the Pasteur group noticed. This placed the new virus within the *Retroviridae* family. Visual analysis of the virions by electron microscopy revealed 100-120μm enveloped virions (Figure 1.6), mature particles contained a cone shaped core which was similar to that of visna virus (Gonda et al., 1985) and its genomic organization placed HIV-1 taxonomically in the Lentivirus genus (Ratner et al., 1985; Wain-Hobson et al., 1985). The Lentivirus genus includes many different viruses which infect a diverse range of animal species causing slow unremitting diseases targeting various lineages of haematopoietic cells (Table 1.2).

Virus	Host infected	Cells with Productive Infection	Clinical Disorder
Equine infectious anemia virus (EIAV)	Horse	Macrophages	Cyclical infection in the first year; autoimmune haemolytic anaemia; sometimes encephalopathy
Maedi/visna virus (MVV)	Sheep	Monocyte/Macrophage lineage and Dendritic cells (Hotzel and Cheevers, 2002)	Encephalopathy/pneumonitis
Caprine arthritis-encephalitis virus (CAEV)	Goat	Monocytes (also epithelial mammary cells, fibroblasts and endothelial cells) (Le Jan et al., 2005)	Immune deficiency; arthritis; encephalopathy
Bovine immune deficiency virus (BIV)	Cow	Macrophages	Lymphadenopathy and lymphocytosis
Feline immunodeficiency disease (FIV)	Cat	T Lymphocytes	Immune deficiency
Simian immunodeficiency disease (SIV)	Primate	T Lymphocytes + Macrophages (Muller and Barre-Sinoussi, 2003)	Immune deficiency and encephalopathy
Human immunodeficiency disease (HIV)	Human	T Lymphocytes and Macrophages (Gorry et al., 2005)	Immune deficiency and encephalopathy

Table 1.2: Members of the Lentivirus genus

This shows the host species and cell type infected with the virus along with the common associated clinical disorders. Both MVV and CAEV are both Ovine Immunodeficiency viruses. Table adapted from (Levy, 1998).

Since previously studied replication competent retroviruses (e.g. MuLVs and HTLVs) were genetically homogeneous it was originally hoped that this would be the case for HIV-1. However, HIV-1's extensive genetic heterogeneity soon became obvious (Benn et al., 1985), especially within the variable domain coding regions of the *env* gene (Starcich et al., 1986; Willey et al., 1986). Even within an infected individual the virus isolates could be highly heterogeneous and therefore the term quasi-species was introduced to define the changing population of virus within the host (Meyerhans et al., 1989).

Initially, phylogenetic analyses (based on *gag* and *env* genes) where carried out on HIV-1 isolates from Europe/North America and Africa, this led to the classification of three major groups: M (major); O (outlier); and N (non-M or O/new) (Simon et al., 1998). The majority of N and O isolates are found in central West Africa e.g. Cameroon and Gabon. Based on complete sequence analysis from isolates of group M, which includes over 95% of the world's isolates, nine discrete clades (A,B,C,D,F,G,H,J and K) were found (Louwagie et al., 1993; Myers, 1994; Robertson et al., 2000) and fifteen major recombinant viruses (Julg and Goebel, 2005). Recombinant viruses, the most well known being the previously designated E now known to be a recombinant between clades A and E (CRF01_AE), are termed circulating recombinant forms. As no full length clade E has been found it is not considered an official clade (Robertson et al., 2000).

Clades of HIV-1 are phylogenetically classified on the basis of 20-50% differences in envelope (*env*) nucleotide sequences. There may be as much as 30-50% difference between the *env* genes of groups M and O and the N group appears to be phylogenetically equidistant from M and O (Figure 1.2) (Gurtler et al., 1994; Simon et al., 1998). Within M subgroups, intra-clade *env* variants differ by 10-15% whereas inter-clade variation of 20-30% is observed (Gao et al., 1998; Murphy et al., 1993).

Phylogenetic analysis of West African HIV-2 strains led to the discovery of seven groups (A-G) (Chen et al., 1997; Gao et al., 1994; Yamaguchi, Devare, and Brennan, 2000) believed to have resulted form seven separate zoonotic transmissions. In 2004 a new strain was proposed as the eighth group (H) for HIV-2 (Figure 1.1), however the nomenclature rules specify that three isolates are required for a new group to be found hence it is currently labelled U or untyped at this time (Damond et al., 2004; Robertson et al., 2000). However, only two, A and B, are known to be pathogenic. This is in contrast to HIV-1 where all known subtypes cause disease. HIV-2 group A is the predominant group circulating in West Africa, including Cape Verde Islands, Gambia, Ghana, Guinea Bissau and Mali (Gao et al., 1994; Marlink, 1996; Peeters et al., 1998; Sarr et al., 2000; Takehisa et al., 1997; Xiang et al., 1997). HIV-2 group B has a slight predominance in Cote d'Ivoire only (Pieniazek et al., 1999). Very few members of each of the groups C-G, have been identified, so they are generally thought to be representatives of unsuccessful, dead-end transmissions from different sooty mangabeys (Chen et al., 1997; Gao et al., 1994; Hunt et al., 1997; Yamaguchi, Devare, and Brennan, 2000). The pathogenicity of groups C-G is unknown.

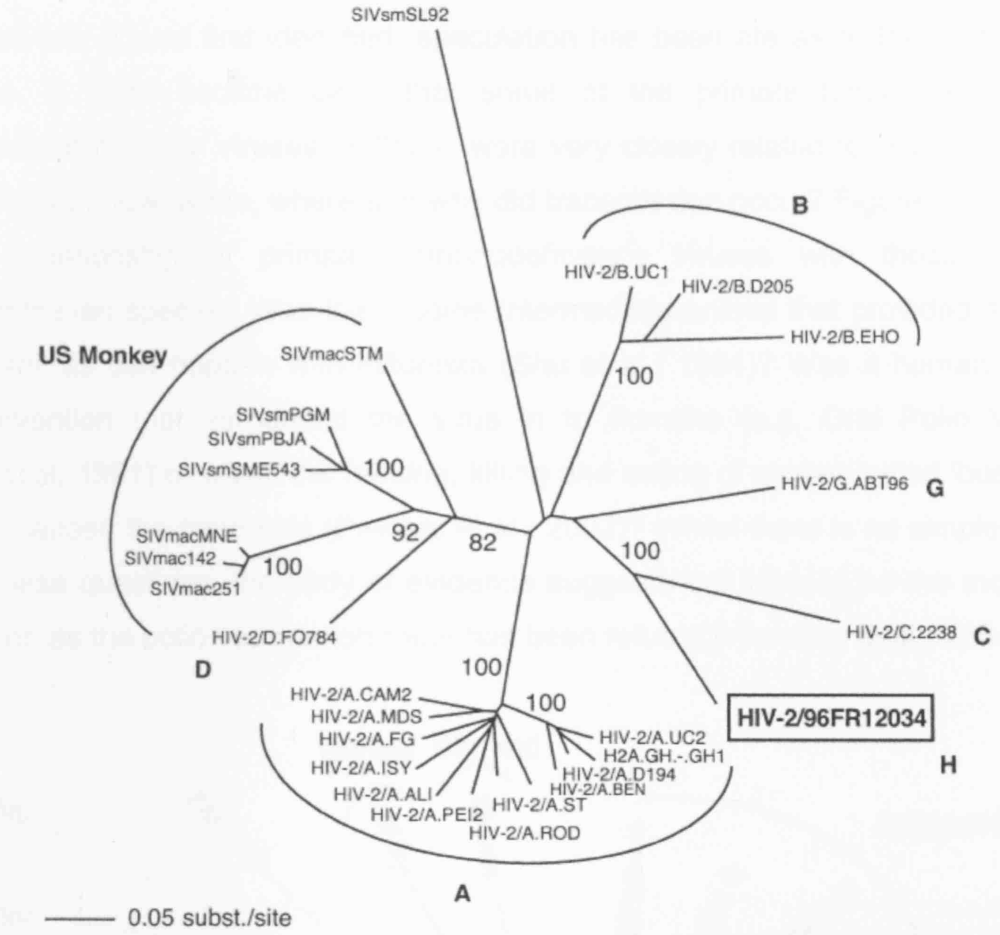


Figure 1.1: Unrooted phylogenetic tree indicating the newly derived HIV-2 strain 96FR12034
Concatenated gag-pol-env fragments of representative HIV-2 (not available for groups E and F) and SIV_{SM} strains were used to generate this maximum likelihood tree. The numbers near nodes indicate the percentage of bootstrap replicates supporting a clade. Bootstrap values greater than 70% are shown. The scale bar indicates substitutions per site and refers to the branch lengths. Adapted from (Damond et al., 2004).

1.3 Origins of HIV

Since HIV-1 was first identified, speculation has been rife as to the origin of the virus. It soon became clear that some of the primate lentiviruses (Simian Immunodeficiency Viruses or SIV's) were very closely related to HIV (Huet et al., 1990), but how, when, where and why did transmission occur? Figure 1.2 indicates the relationship of primate immunodeficiency viruses with those of other mammalian species. Was there some intermediate animal that provided a 'mixing vessel' as can happen with influenza (Shu et al., 1994)? Was it human medical intervention that introduced the virus in to humans (e.g. Oral Polio Vaccine) (Pascal, 1991) or was it the hunting, killing and eating of contaminated 'bush meat' that caused the pandemic (Peeters et al., 2002)? Whilst there is no simple answer to these questions, the body of evidence suggests the latter to be the most likely factor, as the polio vaccination route has been refuted (Worobey et al., 2004).

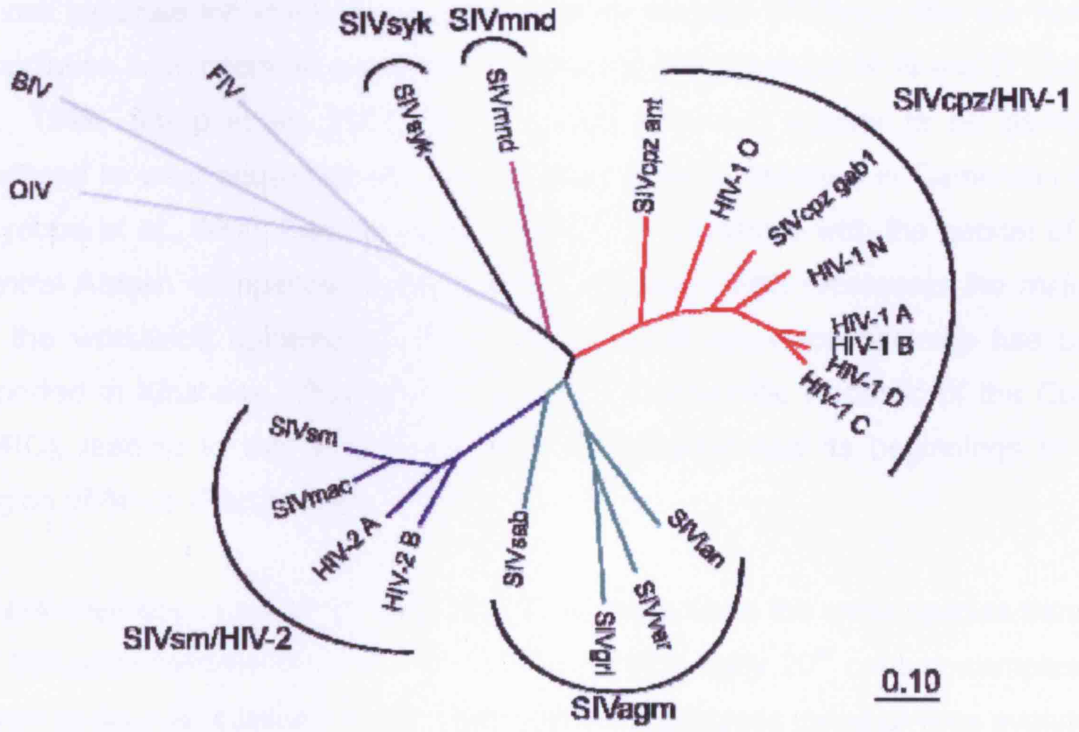


Figure 1.2: The phylogenetic relationships of the Primate Immunodeficiency Viruses.
The tree was constructed using the Maximum Likelihood method based on the full-length pol-gene with a transition-transversion ratio of 1.1. FIV= Feline Immunodeficiency Virus, BIV = Bovine Immunodeficiency Virus and OIV = Ovine Immunodeficiency Virus (MVV was used). The distance bar equates to 10% sequence variation and relates only to the Primate Immunodeficiency viruses shown. The branch for the non-Primate Immunodeficiency viruses has been severely truncated.

1.3.1 HIV-1

As mentioned above (Section 1.3 & Figure 1.2), phylogenetic analysis shows that HIV-1 is closely related to SIV (SIV_{CPZ}) found in the chimpanzee (*P. t. troglodytes*) (Huet et al., 1990; Peeters et al., 1989). However, the chimpanzee is the only species of Ape which appears to be infected with SIV and this virus appears to be a recombinant from two ancestral monkey strains of SIV. The 5' *gag*, *pol*, *vif* and *vpr* genes of SIV_{CPZ} appear to originate from SIV_{RCM} (SIV from the red capped monkeys) whereas the *vpu, env*, *rev* and *tat* of SIV_{CPZ} are derived from SIV_{GSN} /SIV_{MUS} /SIV_{MON} (SIV from the greater spotted nosed, mustached and mona monkeys respectively) whilst the LTR and *nef* are of unknown origin (Sharp, Shaw, and Hahn, 2005). The most likely explanation as to how this occurred is through chimpanzee predation on small monkeys (Chastel and Charmot, 2004), both simian viruses being acquired and recombining within the host to form SIV_{CPZ}.

Three separate introductions or cross species transfer of SIV_{CPZ} into the human population have occurred giving rise to the three HIV-1 groups M, N and O (Gao et al., 1999; Sharp et al., 2001). HIV-1 group N and O appear to be generally confined to west equatorial Africa with group N being reported in Cameroon only (Ayouba et al., 2000; Bodelle et al., 2004). This correlates with the habitat of the central African chimpanzee subspecies. The group which represents the majority of the worldwide epidemic is M yet the greatest sequence diversity has been reported in Kinshasa (Vidal et al., 2000), the Democratic Republic of the Congo (DRC), leading to the assumption that the epidemic had its beginnings in this region of Africa (Rambaut et al., 2001).

There have been several attempts to put a date on when the cross species transfer of SIV_{CPZ} to humans occurred, but without finding early 20^{th} century samples on which to base phylogenetics and molecular clock analyses (rates of virus evolution) all dates put forward are educated guesses. Early studies that estimated the rate of the molecular clock of HIV-1 group M used simplistic methodology and suggested the common ancestor to have been present in 1960 (Li, Tanimura, and Sharp, 1988). More recent studies suggest the virus was introduced earlier than 1960.

One of the main reasons for this is that a blood sample (ZR59) collected in 1959 in the DRC contained a member of the HIV-1 group M clade D lineage after its split from clade B (Figure 1.3) (Zhu et al., 1998). This clearly indicated that the common ancestor must have existed much earlier than 1960 a finding supported by using more complex models to estimate the molecular clock taking into account heterogeneous rates of evolution at different sites within genes, which is the way HIV-1 is seen to evolve (Leitner, Kumar, and Albert, 1997). The most extensive analysis of this question was carried out by Korber and colleagues (Korber et al., 2000) and they estimated the common ancestor of group M to be present in 1931 with a confidence interval of 1915-1941. This was based on HIV-1 *env* sequences from more than 150 individual isolates.

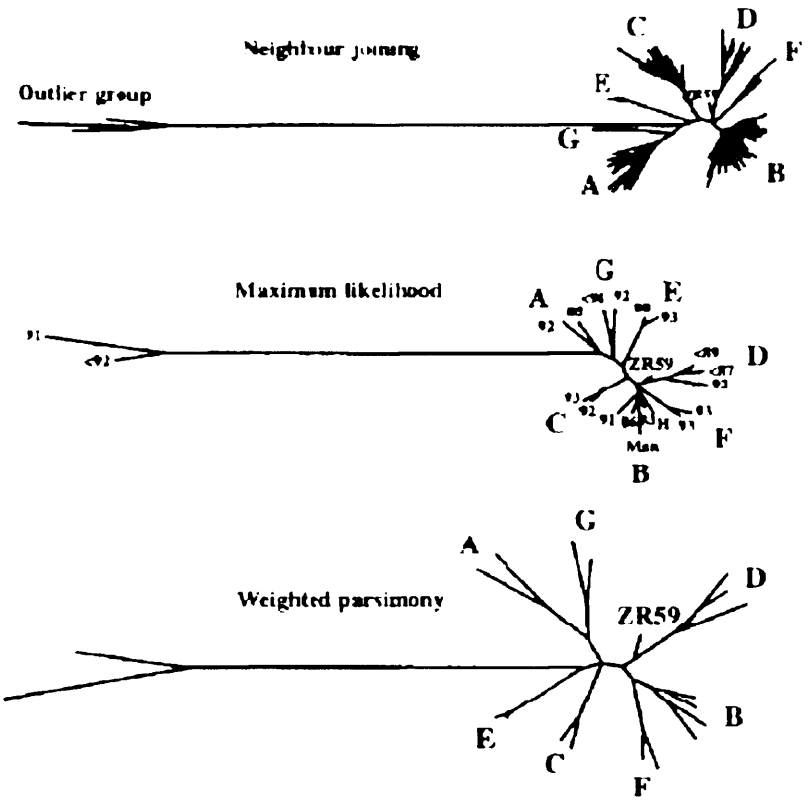


Figure 1.3: Phylogenetic analysis of the *env*-gene of ZR59 sequence

ZR59 branched off the D clade near the B/D/F root using all three phylogenetic methods (Neighbour joining, Maximum likelihood and weighted parsimony). The taxa are labelled with the year of sampling of a given sequence, or with '<' and the year of the primary publication if the year of sampling was not specified. For *env*-gene reference sequences and their GenBank accession numbers see original paper (Zhu et al., 1998). The scale for branch lengths is comparable for the maximum likelihood and neighbour-joining trees. For the weighted-parsimony tree, the branch lengths are not directly comparable to these of the other two trees, but the branching pattern substantiates the pattern obtained by the other two methods.

1.3.2 HIV-2

There is evidence that HIV-2 groups A and B are independent zoonotic transmissions from sooty mangabey to human (Chen et al., 1997). All the criteria for HIV-2 being a zoonosis from the sooty mangabey are met and these are:

- Phylogenetic relatedness (Figure 1.2)
- Similarity in genomic organization (Figure 1.7)
- Geographical overlap (Figure 1.4)
- Prevalence in the natural host (Apetrei, Robertson, and Marx, 2004)
- Plausible route of transmission (Sharp, Robertson, and Hahn, 1995).

The HIV-2 groups can be split into two groups the epidemic strains (A-B) and non-epidemic strains (C-G). Lemey and colleagues (Lemey et al., 2003) used a combination of phylogenetic, molecular clock, and coalescent analyses to provide dates for most recent common ancestors for HIV-2 group A and B which were 1940±16 years and 1945±14 years (95% confidence limits) respectively.

The natural history of HIV-2 in its region of origin is characterised by a period of low endemicity, followed by an exponentially increasing number of infections. It is believed the transition between steady state and exponential occurred between 1955-1970 (Lemey et al., 2003). This time frame coincides with the war for independence (1963-74) in Guinea Bissau (Poulsen et al., 2000) and evidence shows that there was increased sexual and blood-borne transmission throughout this period. Also, massive inoculation campaigns particularly by army-trained doctors at Canchungo are thought to have aided the expansion of the epidemic (Lemey et al., 2003).

Figure 1.4: Historical range of the sooty mangabey (*C. atys*) from south of the Casamance River in Senegal to the Nzo/Sassandra River systems in Cote d'Ivoire

The geographic origin of previously reported SIV_{SM} strains from Sierra Leone, Liberia and western Cote d'Ivoire is shown in relation to the location of the Tai field site. Red dots indicate known capture locations of SIV_{sm}-infected sooty mangabeys, while yellow dots indicate sites where infected sooty mangabeys were identified in captivity (CI2 and CI8 were captured in western Cote d'Ivoire, close to the Liberian border). Green areas depict remaining coastal forest areas (http://www.globalforestwatch.org). Country boundaries and major cities are indicated (Santiago et al., 2005).

1.4 Epidemiology and Transmission of HIV

1.4.1 HIV-1

As with many other epidemics of infectious diseases HIV went unnoticed but, unfortunately, unlike Ebola outbreaks in Africa, HIV infection and transmission was not self-limiting. The UNAIDS estimates for 2005 show that approximately 40 million people worldwide are infected with HIV with five million representing new infections during the course of 2005 (WHO, 2005). Of this 40 million, 3 million died, in 2005, from AIDS or AIDS related diseases and over 0.5 million of these were children under 15 years of age. The majority, at least 26 million, of those infected live in Sub-Saharan Africa, with another 10 million in Asia. In these parts of the world control of a disease like HIV infection is problematic. Superstition and poor education (WHO, 2004) provides a real barrier for control of an epidemic which is

spread through sexual contact or through contaminated blood (Francis, Curran, and Essex, 1983).

More specifically transmission of HIV occurs horizontally from person to person by exposure of the oral, rectal, or vaginal mucosa during sex, through transfusion of contaminated blood products or use of contaminated equipment during injection-drug use (Francis, Curran, and Essex, 1983), or vertically through maternal-foetal circulation, or by breast feeding (Andiman et al., 1990; Hira et al., 1989; Hira et al., 1990). The vast majority of HIV infection is a result of unprotected sex.

Numerous behavioural and biologic factors are required for efficient transmission of HIV, including infection with TB and other sexually transmitted diseases (STD) (Godbole and Mehendale, 2005). The riskiest sexual behaviour appears to be receptive anal intercourse which has a per contact probability of HIV transmission of 0.8% (95% confidence interval 0.24 – 2.76%) (Vittinghoff et al., 1999), whereas it is less than 0.2% probability for male-to-female HIV transmission during vaginal sex (Holmberg et al., 1989; Padian et al., 1997). As with many diseases the genetic composition of the host can alter the probability of HIV transmission (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996) as shown by the wide range of the numbers of exposures required for transmission on a person to person basis (Paxton et al., 1996; Rowland-Jones et al., 1995; Vittinghoff et al., 1999). Other factors that effect transmission are the physical disruption of the exposed mucosa, the size and infectiousness of the inoculum and the local environment in which the exposure occurs (Greenblatt et al., 1988; Quinn et al., 1988; Stamm et al., 1988; Telzak et al., 1993).

1.4.2 HIV-2

In contrast to HIV-1, HIV-2 infection appears to be restricted to West Africa. Large surveys of HIV infected patients in Cameroon (Zekeng et al., 1992), Equatorial Guinea (Zekeng et al., 1997), Gabon (Delaporte et al., 1996; Tevi-Benissan et al., 1998) and the DRC (Mulanga-Kabeya et al., 1998) report specifically that there was no incidence of HIV-2 either individually or as dual infection. They report a few sporadic cases only, suggesting that HIV-2 has not spread to other regions in

Africa. The country with the highest prevalence of disease is Guinea Bissau at 8-10% (Poulsen et al., 1989; Wilkins et al., 1993). In the nearby countries of The Gambia, Senegal and Guinea the prevalence is lower at approximately 1-2% (De Cock et al., 1993). However, commercial sex workers in West Africa can range in HIV-2 or dual positivity from 8.1% in Mali (Peeters et al., 1998) to The Gambia with an incidence of 27.5% (Ghys et al., 1997; Hawkes et al., 1995; Langley et al., 1996).

The epidemic groups A and B of HIV-2 have infected numerous people in West Africa (Damond et al., 2001; Gao et al., 1994; Ishikawa et al., 2001; Pieniazek et al., 1999; Zeh et al., 2005) whereas the other groups discovered, C-G, have each been isolated from an individual patient only (Chen et al., 1997; Damond et al., 2004; Gao et al., 1992; Yamaguchi, Devare, and Brennan, 2000).

A recent study (Santiago et al., 2005) investigating the molecular epidemiology of wild sooty mangabeys has provided the most convincing evidence of the origins of these groups. In particular both the epidemic groups appear to originate from SIV infected sooty mangabeys in the eastern side of their range, the Tai Forest (ranges form eastern Liberia to western Cote d'Ivoire, Figure 1.4). This correlates for group B as the majority of infections have been found in Cote d'Ivoire and neighbouring countries to the east (Ishikawa et al., 2001; Pieniazek et al., 1999). However, it does not correlate for HIV-2 group A which is prevalent in West Africa, represented by countries such as Guinea Bissau (Damond et al., 2001; Gao et al., 1994; Peeters, Toure-Kane, and Nkengasong, 2003). This shows that the area with the most pronounced clinical manifestation of a newly introduced pathogen does not have to coincide with the location of the natural animal reservoir.

The highest incidence of HIV-2 infection outside West Africa is in Portugal, where 13% of all HIV positive sexually transmitted disease patients and 29% of all HIV-positive TB patients are HIV-2 infected (Cazein et al., 1996). Other countries which have incidences of HIV-2 are those with socio-economical links to Portugal such as southwest India (Babu et al., 1993; Kulkarni et al., 1992; Pfutzner et al., 1992; Rubsamen-Waigmann et al., 1991), Angola (Santos-Ferreira et al., 1990),

Mozambique (Barreto et al., 1993) and Brazil (Cortes et al., 1989; Veronesi et al., 1987). In general HIV-2 is uncommon in these countries numbering less than a 100 cases per country (Cazein et al., 1996; Dougan et al., 2005; Nandwani, Shanson, and Coleman, 1994; Sullivan et al., 1998; Toro et al., 2002).

A recent study from the communicable disease surveillance centre (Dougan et al., 2005) in the UK reported on the incidence of HIV-1 and HIV-2 diagnoses, where the patients were likely to have been infected in West Africa. Between 1985-2003 there were 1324 individuals who met these criteria, of these 69% were HIV-1 infected and only 6% were HIV-2 or HIV-1/HIV-2 dually infected, for the remaining 27% the HIV type was not reported. The Gambia was the source of many infections with HIV-2 or HIV-1/HIV-2 dual infections (11.7%-15.2%). They also showed evidence for heterosexual transmission of both HIV-1 and HIV-2 within the UK from people originally infected in West Africa. Although the numbers of people infected with HIV-2 within the UK have risen during the period of this study the levels are still very low in comparison to HIV-1.

Transmission of HIV-2 occurs by the same mechanisms as HIV-1, although blood transfusion is known to be a significant risk factor for infection in Portugal (Cao et al., 1996) and Guinea Bissau (Poulsen et al., 2000). The inequality in the prevalence of HIV-1 and HIV-2 are likely to be attributed to the 'natural' (sexual and vertical) methods of transmission (Kanki et al., 1994; Most et al., 1994). Recent studies (Arien et al., 2005) have investigated the viral fitness of different groups and types of HIV and they discovered that the relative order of fitness is HIV-1 group M> HIV-2> HIV-1 group O. This reflects the order of prevalence of groups and types in the human pandemic.

Theoretically, the efficiency of heterosexual transmission is dependant on the amount of virus in semen or cervico-vaginal secretions (CVS), and the local specific and non specific immunity in the female and male reproductive tracts (Critchlow and Kiviat, 1997). From a study in Cote d'Ivoire (Ghys et al., 1997) approximately 25% of HIV-1 infected women had evidence of the virus in their CVS in comparison to only 4% of HIV-2 infected women. Similar results have been

shown in smaller studies on commercial sex workers (CSW) in Dakar and Senegal (Sankale et al., 1998). These findings may help explain the different transmission rates of HIV-1 and HIV-2 and it is likely that lower shedding of HIV-2 is correlated with a lower plasma viral load in HIV-2 infected women (Alabi et al., 2003).

Established risk factors for sexual transmission of HIV-2 are serological proof of genital ulcer diseases (Pepin et al., 1991; Pepin et al., 1992), a history of STDs (Pepin et al., 1992; Poulsen et al., 1993; Sassan-Morokro et al., 1996; Wilkins et al., 1991), older age (Abbott et al., 1994; Wilkins et al., 1993), having had sex with CSWs (Sassan-Morokro et al., 1996), lack of circumcision in males (Sassan-Morokro et al., 1996), and increased number of lifetime sexual partners (Abbott et al., 1994; Kanki et al., 1992).

Vertical transmission of HIV-2 has been shown in a number of epidemiological studies (Adjorlolo-Johnson et al., 1994; Morgan et al., 1990) which have been demonstrated convincingly in molecular studies (Cavaco-Silva et al., 1997; Cavaco-Silva et al., 1998). Transmission of HIV-2 from an infected mother to her child appears to be a much rarer event than for HIV-1 in the Gambia, 4% compared to 24.4% respectively, this reflects the differences in viral loads in plasma and CVS (O'Donovan et al., 2000).
Studies have shown that HIV-2 transmission can occur via homosexual contact (Cilla et al., 2001; Nandi et al., 1994), but there is a lack of formal studies investigating transmission of HIV-2 via needle stick injuries. However, this remains a plausible method of transmission.

1.5 HIV Pathogenesis

Infection with HIV eventually leads to AIDS, which is commonly associated with infection by opportunistic pathogens. The degree of immunodeficiency and therefore the susceptibility of a HIV infected patient to opportunistic infections can be determined from the $CD4^+$ T cell count (Masur et al., 1989). The CDC in the United States classified the stages of HIV infection and subsequent progression to AIDS based on $CD4^+$ T cell count (Table 1.1). However, the most accurate predictor of disease progression is plasma viral load which can predict the

trajectory of the $CD4^+$ T cell count decline (Figure 1.9) (Mellors et al., 1997; Mellors et al., 1996). HIV infection is characterised by two stages, the early acute stage followed by the chronic stage.

Although it is believed that between 50-90% of primary HIV infection is accompanied by symptoms (Schacker et al., 1996), such clinical indications unfortunately show a marked similarity to many 'flu-like' or 'mononucleosis-like' illnesses (Niu, Stein, and Schnittman, 1993). Further general symptoms which may occur are myalgias, arthralgias, diarrhoea, nausea, vomiting, headache, hepatospleno-megaly, weight loss, thrush and neurologic symptoms. The average duration of symptoms during primary HIV infection is three weeks (Kinloch-de Loes et al., 1993).

The level at which plasma HIV RNA, and therefore viral load, levels peak during this initial phase of infection can vary between thousands and millions of copies per millilitre (Schacker et al., 1998). Usually, there is a decline of plasma viral load at a rate of 6.5% per week for 4 months followed by 5 months of further, more significant, decline (Schacker et al., 1998). However, persistently high levels of HIV RNA at 4-6 months post primary infection, a high viral load set point, may be a predictor of rapid progression to disease (Mellors et al., 1995; Schacker et al., 1998). This primary stage of infection is then followed by an extended period (approximately 10 years) of clinical latency before symptoms of advanced immunodeficiency occur (Figure 1.9).

In several recent studies it has been shown that massive infection and potential irreversible depletion of mucosal-associated lymphoid tissue (MALT) associated $CD4^+CCR5^+$ memory T cells is a defining feature of the acute phase of infection (Brenchley et al., 2004; Guadalupe et al., 2003; Li et al., 2005b; Mattapallil et al., 2005; Mehandru et al., 2004; Veazey et al., 1998; Veazey, Marx, and Lackner, 2003). The decimation of these cells, which are an integral component of the mucosal immunological barrier against invading pathogens, may significantly affect barrier function (Derdeyn and Silvestri, 2005), thereby allowing many sub clinical infections to emerge which leads to micro-environment destruction and chronic

immune system activation. However, this is supposition as opportunistic infections characteristic of full blown AIDS, only occur when blood $CD4^+$ T cell counts decline.

The loss of integrity in cell mediated immune responses occurs when the $CD4^+$ T cell count falls below the level of approximately 200 cells/µl (Table 1.1 and Figure 1.9). At this stage ubiquitous environmental organisms with limited virulence in healthy individuals (e.g. *Pneumocystis carinii* and *Mycobacterium avium*) become life threatening pathogens. These so called opportunistic microorganisms cause HIV-associated illnesses (see Table 1.1) characteristic of full blown AIDS.

As with infection with HIV-1, HIV-2 infection leads to $CD4^+$ T cell decline (Jaffar et al., 1997; Marlink et al., 1994), infection with opportunistic pathogens (De Cock et al., 1991; Grant, Djomand, and De Cock, 1997; Grant et al., 1997; Norrgren et al., 1997), HIV associated malignances (Colebunders et al., 1995; Grant, Djomand, and De Cock, 1997; Matheron et al., 1997), and premature death. The only difference in clinical manifestation between HIV-1 and HIV-2 is that there may be a lower frequency of Kaposi's sarcoma among HIV-2 infected patients (Ariyoshi et al., 1998) and a higher frequency of encephalitis (Lucas et al., 1993).

Intriguingly, infection with HIV-2 is known to lead to a longer period of clinical latency (Ancelle et al., 1987; Marlink et al., 1994) than that seen with HIV-1. There are reported cases with latency periods of 14 years, and even 27 years (Ancelle et al., 1987; Dufoort et al., 1988; Mota-Miranda et al., 1995), such that a large proportion of HIV-2 infected patients could be categorised as long term non-progressors (Lisse et al., 1996).

What determines this longer clinical latency in HIV-2 infected patients is unclear and is probably due to a number of factors such as genetic host factors (HLA type, co-receptor polymorphisms) and mutations or truncations (Grassly et al., 1998; Switzer et al., 1998) of the virus genome. What is known is that the peak of plasma viremia during the acute phase of HIV-2 infection is significantly lower (approximately 30-fold) compared with HIV-1-positive patients and such low levels

are maintained at all stages of HIV-2 infection (Andersson et al., 2000; Popper, 1999; Shanmugam et al., 2000; Soriano et al., 2000). This difference in viral loads between HIV-1 and HIV-2 infected individuals is probably related to the difference in transcriptional regulation (Bock and Markovitz, 2001). Whilst proviral loads for HIV-1 and HIV-2 are similar, as are the levels of latently infected/ non productively infected target cells, the plasma viral load (RNA): provirus load (DNA) rates for HIV-2 are much lower than those for HIV-1 (Popper, 1999; Popper et al., 2000). This is indicative of lower transcription (RNA production) levels for HIV-2.

In terms of HIV-1 infection, it is known that HIV-1 *nef* deletion mutants are non-virulent, and these can be a factor in long-term non-progression of disease (Piguet and Trono, 1999). Switzer and colleagues (Switzer et al., 1998) analysed HIV-2 samples from Cote d'Ivoire, Spain and Portugal and found *nef* deletions in 14% of asymptomatic cases, but in only 4% of AIDS cases. Following this, a study of HIV-2 group A nef sequences, found discrete changes in the PxxP motif correlating with asymptomatic phenotypes (Padua et al., 2003). Further comparative studies are required to determine whether *nef* gene mutations are a significant factor in the lower virulence of HIV-2.

1.6 The Life Cycle of HIV

As with all viruses (obligate parasites) HIV depends on host cells to reproduce and survive. The first step in the replicative life cycle is the interaction between the virus envelope glycoprotein gp120 and the receptor, known to be CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984), which is found on the surface of susceptible cells of the haematopoietic lineage (Figure 1.5). There is then a subsequent interaction, following conformational changes in the viral envelope glycoprotein induced by CD4 binding, with a co-receptor. The co-receptors are known to be members of the seven membrane spanning CC or CXC family of chemokine receptors, the most important being CXCR4 and CCR5. This interaction is required for membrane fusion and virus entry. However, some viruses can infect in a CD4-independent manner through interaction directly with co-receptors (Bhattacharya, Peters, and Clapham, 2003; Thomas et al., 2003). Receptor mediated endocytosis is a

common mechanism of entry for enveloped viruses however HIV-1, HIV-2 and other retroviruses directly fuse their membrane to that of the host cell (Colman and Lawrence, 2003). Partial uncoating of the sub-viral particles occurs in the cytoplasm following entry. This then leads to initiation of reverse transcription of the viral genomes. These early steps in the virus lifecycle following fusion are poorly understood as very high retrovirus particle-to-infectivity ratios (>100:1) hamper experiments (Dimitrov et al., 1993).

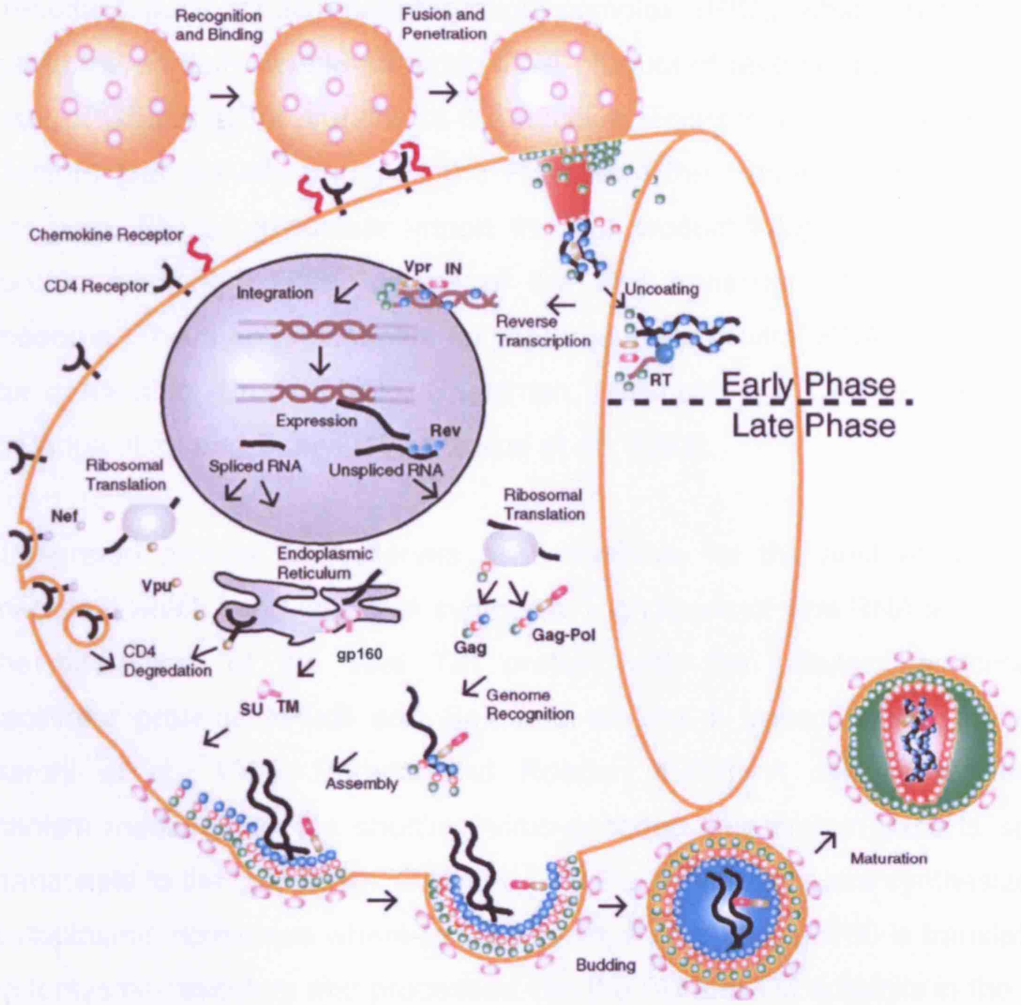


Figure 1.5: Schematic representation of HIV-1 lifecycle

The early phase of the lifecycle is characterised by the penetration of the target cell using a primary receptor (CD4) and co-receptor (a chemokine receptor) at the plasma membrane. Following fusion, penetration and uncoating reverse transcription occurs as the preintegration complex is released into the cytoplasm and transported to the nucleus. The proviral DNA is integrated into the host genome where it can remain latent. Upon activation of the infected cell, the late phase of the lifecycle proceeds with transcription and translation of viral gene products. CD4 is down regulated from the host cell and assembly of the virus occurs at a membrane site, in this case, the plasma membrane. Maturation occurs after budding of the virion. Reprinted from Journal of Molecular Biology, Vol: 285, Turner & Summer, Structural Biology of HIV, pages 1-32, 1999 (Turner and Summers, 1999), with permission from Elsevier.

The resultant nucleoprotein-preintegration complex (PIC), which includes Vpr, transports the partially double stranded DNA product of reverse transcription, with a subset of Gag and Pol proteins to the nucleus. This process is likely to require host factors that actively transport the PICs from the plasma membrane to the nuclear pore. Following nuclear import the Pol product integrase is required to incorporate full length linear copies of the viral transcript into the host cells chromosome. This step is essential for the production of viral RNA and infectious particle production (Brown, 1990; Engelman, Mizuuchi, and Craigie, 1991; Goff, 1992; Panganiban and Temin, 1984; Sakai et al., 1993).

The integrated proviral DNA serves as a template for the host enzyme RNA polymerase II which conducts RNA synthesis. High levels of viral RNA are ensured by the interaction of the viral Tat protein with the cellular transcriptional transactivator proteins NF-κB and Sp1 and the Pol II transcriptional apparatus (Demarchi et al., 1996; Parada and Roeder, 1999). A distinctive transport mechanism mediated by the shuttling virus-encoded Rev protein exports spliced HIV transcripts to the cytoplasm. Gag and Gag-Pol polyproteins are synthesized on free cytoplasmic ribosomes whereas the envelope precursor gp160 is translated in the endoplasmic reticulum and processed into the SU and TM subunits in the Golgi apparatus. The glycoprotein and other structural proteins are then transported by independent pathways to the plasma membrane. An electron dense 'bud' is formed at the plasma membrane this is caused by the Gag, Gag-Pol polyproteins and dimers of genomic RNA being closely associated. A spherical immature particle containing the mature TM and SU envelope glycoproteins is released from the cell. Mature virions are formed during or immediately after particle release, containing a characteristic cone-shaped nucleoid formed by proteolytic processing of the Gag and Gag-Pol proteins by the HIV protease (Craven and Parent, 1996; Shehu-Xhilaga et al., 2002) (Figure 1.6).

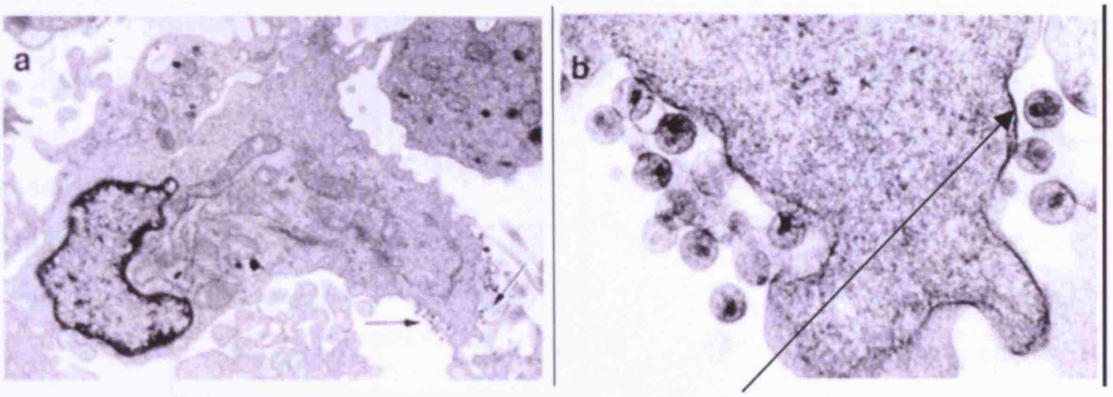


Figure 1.6: Electron Micrograph of HIV virions

(a) Transmission electron micrograph of an activated monocyte showing a pseudopod with associated (strain O/S) virions (arrows). (b) Higher magnification of the cell shown in panel a showing mature HIV virions associated with the pseudopod. Magnification, x 3,135 (a); 38,950 (b). Permission has been granted by both the American Society for Microbiology and from the corresponding author Dr David Phillips to use these micrographs (Perotti, Tan, and Phillips, 1996).

1.7 Treatment of HIV infection

1.7.1 HIV-1

There are a number of distinct stages during the viral life cycle which are, or could be, targeted by drugs or vaccines to slow down or inhibit disease progression. As the enzyme reverse transcriptase (RT) is unique to the virus (i.e. not present in the host) (Jeffries, 1989) RT targeted drugs were the first to become available (Mitsuya et al., 1985) and they are still essential today in the treatment of HIV infection. There are two types called nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). Other drugs used in anti-retroviral therapy are protease inhibitors (PI) and fusion inhibitors. There are more possible targets for which drugs are in development and these include inhibitors of entry, HIV integrase and nucleocapsid zinc-fingers (Reeves and Piefer, 2005; Saksena and Haddad, 2003). There are currently 18 drugs which are approved for the treatment of HIV infection (Table 1.3), the different classes of which will be discussed below.

Class	Drugs	Abbreviation	Viral Target	Mode of Action
Nucleoside reverse transcriptase inhibitors (NRTIs)	Zidovudine	AZT	Reverse transcriptase	Phosphorylated by cellular enzymes. Competitively inhibits viral DNA synthesis by causing chain termination. TFV is a nucleotide analogue
	Didanosine	ddI		
	Zalcitabine	ddC		
	Stavudine	D4T		
	Lamivudine	3TC		
	Abacavir	ABV		
	Tenofovir	TFV		
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Nevirapine	NVP	Reverse transcriptase	Not phosphorylated. Non-competitive inhibition of viral DNA synthesis. Binds directly to enzymes
	Delavirdine	DLV		
	Efavirenz	EFV		
Protease Inhibitors (PIs)	Saquinavir	SQV	Protease	Binds to protease active site, thereby inhibiting enzyme function
	Indinavir	IDV		
	Ritonavir	RTV		
	Nelfinavir	NFV		
	Amprenavir	APV		
	Lopinavir	LPV		
	Atazanavir	ATN		
Fusion Inhibitors	Enfuvirtide	T-20 (peptide)	Envelope gp41	Binds to N-terminal α-helix region of the gp41 envelope glycoprotein

Table 1.3: Different classes of licensed HIV-1 antiretroviral agents

Reprinted by permission from the Indian Journal of Medical Research, Vol: 119, Issue:6, pages 217-37, Potter, S.J., Chew, C. B. Steain, M. Dwyer, D.E. and Saksena, N. K. Copyright 2004 the IJMR (Potter et al., 2004).

The identification of CD4 as the receptor for HIV on T cells (Dalgleish et al., 1984; Klatzmann et al., 1984) led to the idea of a therapeutic use for soluble CD4 (sCD4) to 'mop up' virus before they could infect cells (Schooley et al., 1990). While laboratory strains of HIV-1 were efficiently neutralised by sCD4 products, primary isolates were not (Matthews, 1994). Enhanced infection rather than inhibition was observed with primary isolates as gp120 was shed from virions in the presence of sCD4 exposing the fusogenic ectodomain of gp41 (Berger, Lifson, and Eiden, 1991; Bour, Geleziunas, and Wainberg, 1995).

The viral enzyme RT controls the production of viral DNA prior to integration into the host cell genome. The HIV-positive sense RNA is used as a template, against which the RT catalyses the assembly of deoxynucleoside triphosphates (dNTPs), hence forming a negative sense DNA strand. The RT also has a ribonuclease activity causing the degradation of the positive sense RNA from the negative sense DNA, and a DNA polymerase activity allowing the formation of a second positive-sense DNA strand, thereby producing the double stranded proviral DNA (Tarrago-Litvak et al., 1994). NRTIs structurally resemble endogenous dNTPs and compete with them for binding to the RT active site. Once incorporated into the growing HIV DNA strand, their modified 3' hydroxyl group causes chain termination of DNA synthesis. NNRTI are a diverse group of compounds in a structural sense. Nevertheless, they all have an aromatic structure which binds to the hydrophobic pocket near the polymerase site of HIV-1 RT, preventing the enzyme's function.

The protease of HIV-1, HIV-2 and SIV, is required to produce a mature retrovirus, and belongs to the aspartyl protease family. It is required for the post-translational processing and cleavage of the polyprotein products Pr^{GAG} and $Pr^{GAG\text{-}POL}$ into functional core proteins and viral enzymes, such as reverse transcriptase, integrase and protease. The inhibitors of this enzyme function by competitively binding to the protease substrate site. This results in the production of immature, non-infectious particles (Lewis et al., 1997).

More recent advances in retroviral therapy have related to the introduction of an entry inhibitor, enfuvirtide (T-20). T-20 prevents fusion between the virus and host cell membranes by binding to a portion of gp41 preventing 6 helix bundle formation (Figure 1.18) (Wild, Greenwell, and Matthews, 1993).

Despite this armoury of drugs to combat the virus, infection is not cleared and resistance eventually develops. Resistance to NRTI drugs occurs when base changes in the RT gene result in certain amino acid changes in the protein which affect the tertiary structure of the functional regions of the enzyme, particularly the active site (Isel et al., 2001). The most common mutation is K103N. This and other single mutations located at or near the binding pocket can lead to resistance to NNRTIs (Soriano and de Mendoza, 2002). Although resistance of HIV-1 to protease inhibitors is not completely understood, mutations within the protease gene can confer resistance to specific drugs, e.g. D30N induced by nelfinavir and L90M induced by saquinavir (Temesgen and Wright, 1999). Similarly, there is evidence that HIV-1 will be able to resist fusion inhibitors (T-20) by mutations in the HR1 and HR2 regions of the ectodomain of gp41. In order to slow down the development of resistance, drugs are often used in combination to attack the virus at different stages of the life cycle; this method of treatment is called highly active antiretroviral therapy (HAART) (Collier et al., 1996).

1.7.2 HIV-2

Although HIV-1 and HIV-2 are similar, differences in their reverse transcriptases and proteases result in different susceptibility to the drugs developed for HIV-1. There are a limited number of studies looking at treatment regimes for HIV-2 infected patients and even fewer comparing treatment between HIV-1 and HIV-2 infected individuals as the majority of those infected with HIV-2 live in West Africa where antiretroviral treatment is limited and at best sporadic.

It would appear from both *in vitro* and *in vivo* studies that NRTIs inhibit both wild type and mutant HIV-2 clones (Perach, Rubinek, and Hizi, 1995) and the concentrations required to inhibit wild type HIV-2 are similar to those for HIV-1 (Balzarini et al., 1997; Balzarini et al., 1996; Coates et al., 1992; Cox et al., 1994;

Korba and Milman, 1991). Studies have shown that treatments including NRTIs decrease HIV-2 viral load, increase $CD4^+$ T cell counts and improve AIDS-related symptoms (Cox et al., 1994; Matheron et al., 2003; Rodes et al., 2005).

The other RT group of drugs, NNRTIs, have no effect on HIV-2 *in vitro* which is probably due to differences in the secondary structure of the two RT NNRTI binding pockets (Hizi et al., 1993). A recent study showed the 50% effective concentration (EC_{50}) of various NNRTIs against HIV-2_{ROD} to be at least 2000 fold higher than that of the EC_{50} of the same drugs against HIV-1_{IIIB} (Witvrouw et al., 2004). Despite being a very safe drug when used against HIV-1, the concentration required for NNRTIs to be effective against HIV-2 is toxic making them a questionable choice for therapy against HIV-2 infection (Buckheit et al., 2001; Witvrouw et al., 1999).

Kinetic studies of protease inhibitors (PIs) have indicated that this class of drugs is effective against HIV-2, but binding to the HIV-2 protease is 10-100 fold less efficient, depending on the inhibitor (Pichova et al., 1997; Tomasselli et al., 1990). Witvrouw (Witvrouw et al., 2004) studied five protease inhibitors and their EC_{50} for three laboratory adapted strains of HIV (HIV-1_{IIIB}, HIV-2_{ROD} and HIV-2_{EHO}). Three of the inhibitors had similar EC_{50}s for all strains, however Nelfinavir and Amprenavir had a 2-14 fold increase in EC_{50} values for HIV-2 strains. There are reports that inclusion of PIs in therapy regimes for HIV-2 infection results in a decreased viral load, increased $CD4^+$ T cell count and an improvement in AIDS related symptoms (Smith et al., 2001).

Concentrations of enfuvirtide required to reduce the HIV-2 cytopathic effect to 50% in MT-4 cells were at least 60 times that of the concentration required for the drug to be effective against HIV-1 (Witvrouw et al., 2004). This is due to its specificity for the HIV-1 envelope protein. In the same study an entry inhibitor (AMD 3100) that acts as a CXCR4 antagonist works equally well against HIV-1 and HIV-2. Such a CXCR4 antagonist may be useful in the treatment of HIV-2 infection.

Resistance of HIV-2 to available drugs develops in similar ways to those seen for HIV-1 infection. Antiretroviral therapy can only control the virus for a limited period of time. However, it is apparent from the above studies that within the collection of antiretroviral drugs designed for HIV-1, a proportion are effective against HIV-2. From such studies and from the limited reports of treatment of dual infection (Mullins et al., 2004; Rodes et al., 2005; Schutten, van der Ende, and Osterhaus, 2000; Smith et al., 2001) it is clear that careful consideration should be given to the treatment regime, on diagnosis of HIV-2 infection.

1.8 Genomic Organisation of HIV-1 and HIV-2

The genome organisation of HIV-1/SIV_{CPZ} and HIV-2/SIV_{SM} are shown in Figure 1.7 and the functions of individual gene products are given in Table 1.4.

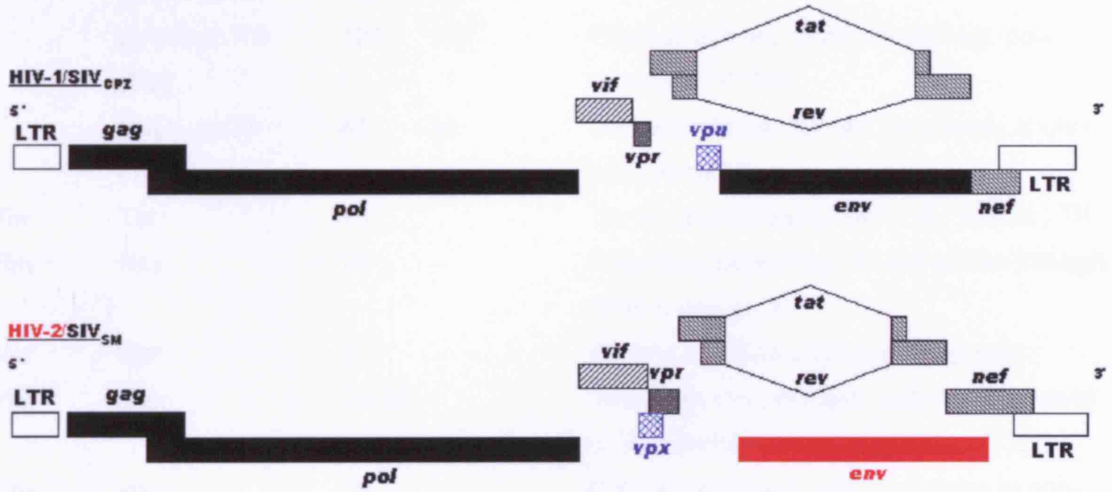


Figure 1.7: Genomic maps of HIV-1 and HIV-2

The 9.8Kb proviral genomes of HIV-1 and HIV-2 show similar patterns of organisation. 5' and 3' Long Terminal Repeat (LTR) regions flank the genomes. The structural genes, *gag*, *pol* and *env* are in similar positions shaded black and red (highlighted HIV-2 *env*). The accessory genes *vif*, *vpr*, *tat*, *rev* and *nef* are common to both viruses. *Vpu* is unique to HIV-1/SIV_{CPZ} and *Vpx* to HIV-2/SIV_{SM}.

Gene	Protein	Molecular weight (kDa)	Function
Gag	Pr55Gag	55	Gag polyprotein precursor
	MA	17	Matrix protein, lines inner surface of virus membrane
	CA	24	Capsid protein, forms virus core
	NC	7	Nucleocapsid protein, binds vRNA
Pol	Pr160Gag-pol	160	Gag-Pol polyprotein precursor
	PR	10	Protease, cleaves Gag and Gag-Pol polyprotein precursors
	RT	66/51	Reverse transcriptase, copies RNA genome to produce proviral DNA for integration
	IN	31	Integrase, integrates proviral genome into host genome
Env	HIV-1 HIV-2	HIV-1 HIV-2	Envelope polyprotein precursor
	gp160 gp140	160 140	
	gp120 gp105 (SU)	120 105	External domain, virion attachment, down regulation of CD4
	gp41 gp36 (TM)	41 36	Transmembrane domain membrane anchor, membrane fusion
Tat	Tat	16	Transactivates transcription via TAR in LTR
Rev	Rev	19	Regulates expression of viral mRNA through RRE in *env* gene
Vpr	Vpr	15	Permits infection on non-dividing cells
Vpu	Vpu	15-20	Influences virus release, augments turnover of CD4 antigen, down regulation of CD4
Vpx	Vpx	16	Critical for efficient virus replication in non-dividing cells
Vif	Vif	23	Helps to overcome host intracellular defence mechanism
Nef	Nef	25-27	Enhances infection, down regulates CD4 & MHC-I expression and modulates T-cell activation

Table 1.4: Genes and Proteins of primate lentiviruses

1.8.1 HIV-1

HIV-1 has a vRNA positive sense genome that is transcribed to produce a 9.8Kb dsDNA proviral genome containing a number of unspliced and spliced gene fragments (Figure 1.7). The three main open reading frames are *gag*, which contains the structural genes, *pol*, coding for the viral enzymes necessary in the life cycle and *env*, encoding the precursor envelope glycoprotein gp160. HIV replication is controlled by the gene products of other spliced mRNAs which produce a number of viral regulatory and accessory proteins (Figure 1.7 and Table 1.4).

The full length mRNA (~9Kb) is translated into the Gag and Pol proteins. Proteolytic processing of the Gag precursor p55 allows the production of matrix (p17), capsid (p24), nucleocapsid (p7) and p6. Cleaved products of the Pol precursor protein are the viral enzymes protease, RT and integrase. Studies have shown that the Gag and Gag-Pol products are synthesized in a ratio of 20:1 (Oroszlan and Luftig, 1990). The viral protease processes the Gag and Pol polyproteins and integrase (IN) is involved in viral DNA integration into the host genome. Integrase has three functions, two occur as part of the integration process, 3'-end processing and strand transfer linking viral and cellular DNA, the third function, termed the disintegration reaction, allows viral DNA to be cleaved from target DNA (Chiu and Davies, 2004).

Singly spliced mRNAs (~4Kb) encode the Vif, Vpr, Vpu and Env proteins with the latter two being on the same, bicistronic, mRNA. As mentioned above the *env* gene encodes the precursor gp160 and this precursor is subsequently cleaved, most likely by furin-like enzymes (Hallenberger et al., 1997; Moulard et al., 1999), to produce the transmembrane envelope glycoprotein (gp41/TM) and the surface envelope glycoprotein (gp120/SU).

Vif (viral infectivity factor) (von Schwedler et al., 1993), Vpr (viral protein r) and Vpu (viral protein u) are termed the accessory proteins. They are known to affect viral functions such as assembly and budding, as well as infectivity during the

production of infectious virus. Some research has indicated that *vpr*, *vpu* and *nef* may be more important for replication of HIV-1 in macrophages than in $CD4^+$ lymphocytes (Anderson and Hope, 2004; Balliet et al., 1994).

Vif is important for production of highly infectious mature virions. This 192 amino acid protein appears to function before or during DNA synthesis as Vif deficient virus exhibits a marked reduction in the levels of DNA synthesis and produces highly unstable replication intermediates (Cohen, Subbramanian, and Gottlinger, 1996; Simon and Malim, 1996). However, these mutants only show these characteristics in certain cell types, termed non-permissive or semi-permissive but not in permissive cell types. More recently, a human protein has been identified in non or semi permissive cells, called hA3G (formerly CEM15) a member of the human apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like (APOBEC) family, which is capable of potently suppressing the infectivity and replication of *vif*-deficient HIV (Sheehy et al., 2002). APOBEC acts by attacking newly made HIV RNA or DNA: cysteines are changed to uracil in viral RNA or guanosine to adenosine in viral DNA resulting in non-active forms of the HIV genome (Harris et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Zhang et al., 2003). When Vif is present it induces ubiquitinisation and subsequent proteasome degradation of hA3G (Marin et al., 2003; Mehle et al., 2004; Sheehy, Gaddis, and Malim, 2003; Yu et al., 2003; Zhang et al., 2003).

Following viral 'uncoating' in the cytoplasm of the host cell, Vpr mediates transport of nucleoprotein complexes to the nucleus (Cohen, Subbramanian, and Gottlinger, 1996). These complexes remain undefined, however they do include RT, IN and MA (Miller, Farnet, and Bushman, 1997) and definitely the genomic RNA and the partially reverse-transcribed DNA. The 96-residue Vpr protein is particularly important in CCR5 M-tropic virus infection of non-dividing cells, as it has a nuclear localisation signal (NLS) which can direct transport in the absence of mitotic nuclear envelope breakdown (Cohen, Subbramanian, and Gottlinger, 1996). Vpr can also induce G2 cell cycle arrest prior to nuclear envelope breakdown and chromosome condensation, and sustained expression of it can reportedly kill T cells by apoptosis (Emerman, 1996). Other proposed functions for Vpr are that it

can decrease mutation rates (four fold) during viral DNA synthesis (Mansky, 1996) and form an ion channel (Lamb and Pinto, 1997).

Vpu is an oligomeric integral membrane protein of 81 amino acids, the N-terminal has 24 hydrophobic amino acids which from a membrane spanning domain whilst the C-terminal forms a cytoplasmic tail (Cohen, Subbramanian, and Gottlinger, 1996; Lamb and Pinto, 1997). Vpu causes CD4 degradation (Bour, Schubert, and Strebel, 1995) by inducing phosphorylation of Ser 52 and 56 of CD4 (Cohen, Subbramanian, and Gottlinger, 1996), thereby preventing the sequestration of Env proteins in the endoplasmic reticulum. Additionally Vpu can down regulate MHC class I proteins, thereby protecting infected cells from CTL mediated cell apoptosis (Kerkau et al., 1997), enhance viral release (Gottlinger et al., 1993), and it has been proposed to form an ion channel (Deora, Spearman, and Ratner, 2000; Lamb and Pinto, 1997).

The regulatory proteins Tat, Rev and Nef are encoded by doubly spliced mRNAs (~2Kb) with the latter two being translated off a bicistronic mRNA. These proteins are produced in the early phase of the virus lifecycle.

The transactivating protein (Tat) along with certain cellular proteins, binds to an RNA loop structure called the Tat-responsive element (TAR), in the 5' long terminal repeat (LTR) of the HIV genome. Tat is required to upregulate HIV and target cell replication (Dayton et al., 1986; Fisher et al., 1986; Pugliese et al., 2005).

The regulator of viral protein expression or Rev protein is required to determine the relative amounts of unspliced to singly and multiply spliced mRNAs. Rev is itself a product of multiply spliced mRNA (Feinberg et al., 1986; Sodroski et al., 1986). It is able to carry out its function by interacting with the Rev-response element (RRE) which is a *cis*-acting RNA loop structure located in the viral envelope mRNA (Feinberg et al., 1986; Sodroski et al., 1986). Multimers of the Rev protein along with cellular proteins are required for this interaction which allows unspliced, and singly spliced mRNA to enter the cytoplasm from the nucleus and give rise to full-length viral proteins necessary for progeny production (Emerman, Vazeux, and

Peden, 1989; Li et al., 2005a; Malim et al., 1989). Hence, Rev seems to affect the activity of spliceosomes.

Negative factor or Nef was given its name due to the protein's ability to down regulate viral expression. However, Nef is not fully understood and a variety of other functions have been attributed to it. These include cell activation and interactions with cellular proteins to cause signal transduction (Sawai et al., 1994). Nef is capable of down-regulating cell surface expression of several cellular proteins (including CD4, CD8, CD28, major histocompatibility complex class I and class II proteins) but it upregulates the invariant chain of MHC II (CD74) (Schindler et al., 2003). There effects disturb the proper functions of the immune system and therefore have profound effects of anti-HIV immune responses Nef's function of interfering with cellular signal transduction pathways causes the production of chemokines which attract T cells and promote their infection by HIV. Nef also remodels actin and can therefore facilitate movement of the viral core past an actin barrier and hence enhance virion infectivity and viral replication (Li et al., 2005a). Cholesterol can be bound by Nef and transported to the site of viral budding enhancing HIV-1 particle production (Zheng et al., 2003).

1.8.2 HIV-2

At the nucleotide sequence level, HIV-2 is closely related to SIV_{SM} but different to HIV-1, showing 75% and 40% similarity respectively (Chakrabarti et al., 1987; Guyader et al., 1987; Hirsch et al., 1989). However, there is a high degree of homology between HIV-1 and HIV-2 in terms of eight of the gene products produced. As for HIV-1, Gag contains matrix, capsid, nucleocapsid and p6, Pol contains protease, RT and integrase and Env the precursor envelope glycoprotein gp140 (Table 1.4). They only vary slightly in the size of the proteins e.g. HIV-1 SU envelope glycoprotein has an apparent molecular weight of 120kDa whereas its equivalent in HIV-2 is 105kDa.

There are differences within the accessory genes. Both Vif and Vpr protein products are coded for in the HIV-2 genome. However, *vpu* is absent from the HIV-2 genome. There is a new coding frame which produces a 15kDa protein termed

Vpx. This gene is found throughout members of the HIV-2/SIV_{SM}/SIV_{MAC} lineage of primate Lentiviruses. *vpx* and *vpr* show significant relatedness so it was initially suggested that *vpx* was created by a *vpr* gene duplication event after the HIV-2/SIV_{SM}/SIV_{MAC} lineage diverged from other primate Lentiviruses (Figure 1.2) (Tristem et al., 1990). More recent studies have proposed that *vpx* was acquired from the SIV_{AGM} *vpr* gene by either non-homologous (Sharp et al., 1996) or homologous (Tristem, Purvis, and Quicke, 1998) recombination.

Both Vpr and Vpx are incorporated into virus particles at high levels, *via* an interaction with the C-terminus of Gag (Henderson et al., 1988; Kewalramani and Emerman, 1996; Wu et al., 1994). Intriguingly, the major functions of HIV-1 Vpr are shared between the Vpx and Vpr in the HIV-2/SIV_{SM}/SIV_{MAC} viruses. Vpr induces cell cycle arrest (Fletcher et al., 1996) whereas Vpx is required for efficient infection of non-dividing cells (Yu et al., 1991).

1.9 Virion Morphology and Structure

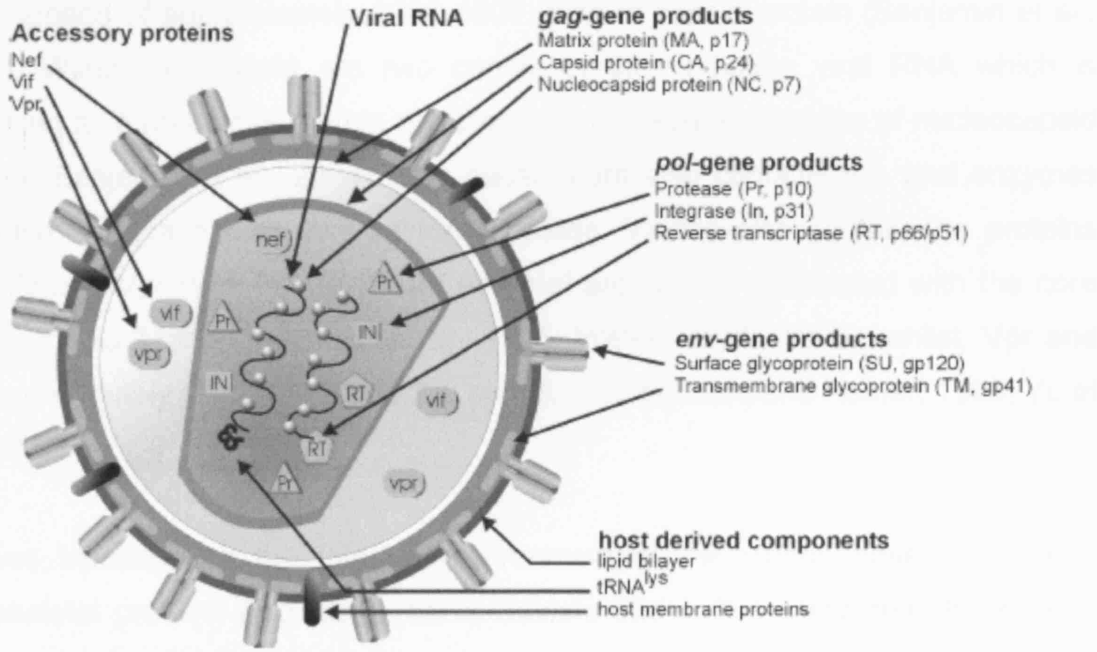


Figure 1.8: The Virion Structure of HIV

The virion of HIV consists of a host cell derived envelope into which the HIV envelope glycoproteins are inserted. The matrix protein (p17) is assosciated with the inner surface of the viral membrane. Contained within the matrix, a capsid core composed of p24 encloses the viral genome.

Electron microscopy of HIV-1 and HIV-2 shows a characteristic Lentivirus with a cone shaped core composed of the viral capsid protein (p24) (Figure 1.6). The mature virion is coated in a lipid bilayer termed the envelope which is derived from the host cell membrane. Proteins which project from the surface of the envelope are the virus transmembrane (TM) subunit which forms non-covalent interactions with the surface glycoprotein (SU) to form spikes on the surface of the virus. Electron microscopy (Ozel, Pauli, and Gelderblom, 1988) evidence and information from the crystal structure of the ectodomain of the TM (Chan et al., 1997; Weissenhorn et al., 1997b) indicate these form trimers. The lipid bilayer also contains several cellular membrane proteins derived from the host cell, including major histocompatibility antigens, actin and ubiquitin (Arthur et al., 1992) and possibly the viral protein Vpu (Montal, 2003). A shell of approximately 4000 - 5000 units of myristoylated matrix protein (Benjamin et al., 2004), which is vital for the integrity of the virion, lines the inner surface of the viral envelope (Gelderblom, Ozel, and Pauli, 1989). The MA protein is required for incorporation of Env proteins into virus particles (Yu et al., 1992). In the centre of the virus the cone shaped core is composed of approximately 1000-1500 units of capsid protein (Benjamin et al., 2004). Within the capsid are two copies of the complete viral RNA which is stabilised as a ribonucleoprotein complex with thousands of copies of nucleocapsid protein (Benjamin et al., 2004). The capsid core also contains the viral enzymes protease, integrase and reverse transcriptase. Virions also contain the proteins Nef, Vif and Vpr (Vpx in HIV-2). Vif and Nef are closely associated with the core (Camaur and Trono, 1996; Liu et al., 1995; Welker et al., 1996), whilst, Vpr and Vpx are probably situated outside the core (Lu, Spearman, and Ratner, 1993; Yu et al., 1993).

Studies investigating the internal environment of the virions have also found cytoskeletal proteins (e.g. actin, ezrin, moesin and coflin) which may have been cleaved by the HIV-1 protease. The role of these cellular proteins in infection, if any is unknown (Ott et al., 1996). However, the host derived tRNA is used as a primer by the virus to initiate reverse transcription (Tisne, 2005).

The major sites for antibody mediated neutralisation are found on the exposed SU (gp120/gp105) and ectodomain of TM (gp41/gp36) portions of the envelope glycoprotein (Nabel, 2005).

1.10 Immunological Response to HIV infection

The typical course of disease progression following infection with HIV-1 is shown in Figure 1.9.

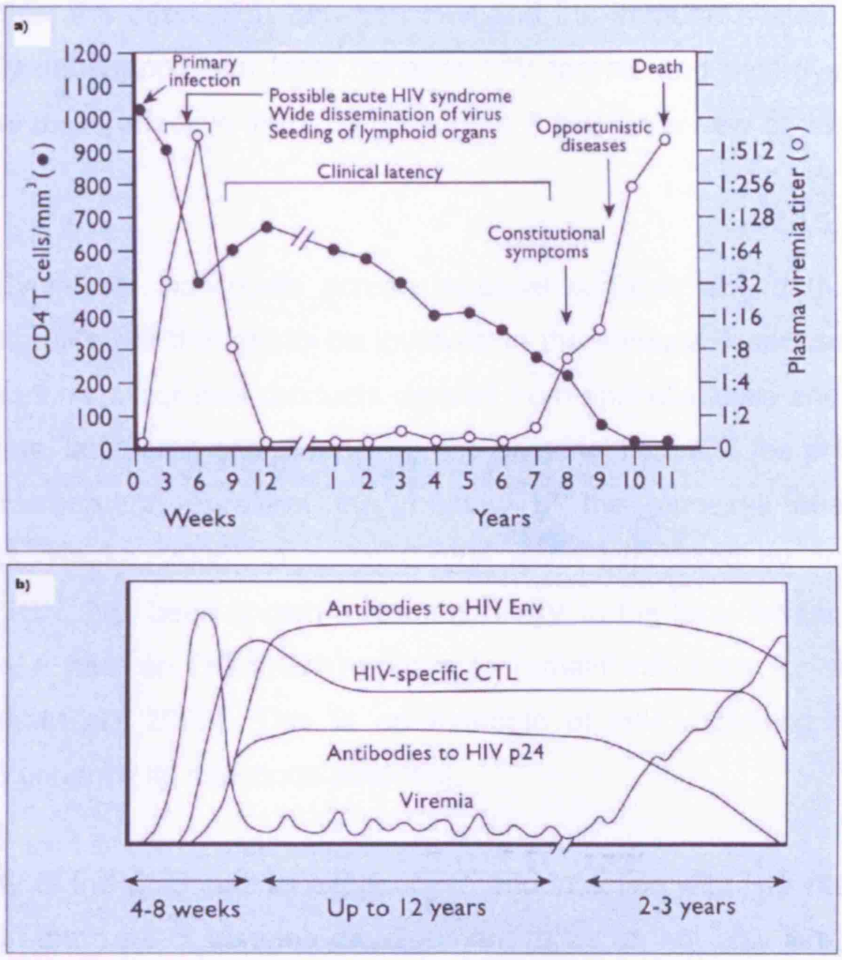


Figure 1.9: Model of the expected course of disease progression following initial infection with HIV-1

a) Natural history of HIV infection and the immune response (adapted from Pantaleo with permission from The New England Journal of Medicine (Pantaleo, Graziosi, and Fauci, 1993)). b) Typical clinical course of HIV infection (Reprinted with permission from Weiss (Weiss, 1993). Copyright 2006 AAAS). The median time from seroconversion to AIDS-related death is approximately 10 years, assuming the absence of anti-retroviral therapy. The clinical latency period persists until $CD4^+$ T lymphocyte numbers fall below 500 cells/mm^3 and viral load in the blood increases. AIDS manifests when cell numbers fall below 200 cells/mm^3, rendering the patient susceptible to opportunistic pathogens characteristic of AIDS.

1.10.1 HIV-1

The human immune system is incredibly complex and our understanding of it, which is continually being updated as new data becomes available, is incomplete. Further to this, the interaction between HIV and the immune system is complex and not fully understood, not least because HIV targets and destroys a range of cells integral to an effective immune response. A brief overview of what is known follows.

HIV normally infects individuals across mucosal surfaces and both innate and adaptive immunity are thought to be involved in the immune responses to HIV at these surfaces. Antimicrobial products derived from epithelial cells and neutrophils (e.g. lysozyme, lactoferrin and defensins), the bacterial flora and the pH of the local mucosal environment represent the majority of the mucosal innate defense mechanisms (Haase, 2005). A novel C-type lectin expressed by dendritic cells (DC), DC-SIGN, has been shown to transport HIV to the local lymph nodes. HIV that 'hitches a ride' on DC-SIGN appears to remain infectious for several days (Geijtenbeek et al., 2000). This is an example of HIV exploiting the immune response to enhance its infectious potential.

The diversity of the response to exposure to and infection with HIV makes it clear that infection and rate of disease development relies on not only viral factors but also the genetics of the host. The end result of infection with HIV is the severe and consistent depletion of $CD4^+$ T cells. HIV achieves this by multiple mechanisms including apoptosis, formation of syncytia and destabilisation of host cell membranes.

HIV Env may cause apoptosis by cross linking CD4 molecules (Banda et al., 1992) and Nef protein in the extracellular matrix can induce cell death in uninfected T cells (Azad, 2000). Both Fas-mediated and Fas-independent apoptosis are upregulated by Tat expression (Peruzzi, 2006). Increased cellular apoptosis correlates directly with disease progression and inversely with T helper cell counts (Fowke et al., 1997; Gougeon et al., 1996).

Syncytia form when CXCR4 tropic HIV isolates preferentially infect T cells and induce membrane fusion between adjacent cells which causes the formation of a giant multinucleate cell. Syncytia are a feature of late stage disease affecting infected and non-infected cells (Alimonti, Ball, and Fowke, 2003).

It is generally believed that host cell membranes are weakened by the continuous budding of infectious viruses with the associated instability leading to death of the cell (Fauci, 1988). Further, through the adaptive immune response HIV infected cells are recognized by HIV-specific $CD8^+$ cytolytic T lymphocytes (CTL) through a T cell receptor in a HLA restricted manner. This contact produces a series of events which leads to the lysis of the infected host cell. Overall, given the range of immune cells that HIV infects and their loss, a gradual decline in the patient's innate and adaptive immunity occurs (Alimonti, Ball, and Fowke, 2003).

There are many host factors which can affect rates of disease progression. Rapid or slow progression to disease may be determined by the host's HLA-I make-up. Certain alleles (e.g. B57, B14, C8 and B27), have been associated with a long term non-progressor status (Hendel et al., 1999; Klein et al., 1998; McNeil et al., 1996), whilst others (HLA B3501 and Cw4) present consistantly in patients showing rapid progression (Tomiyama et al., 1997). Overall, it appears that control of viremia and slower disease progression correlates with heterozygosity at HLA loci presumably due to the breadth of the adaptive immune responses generated (Carrington et al., 1999).

Mutations within the genes for CCR5 or CXCR4 appear to affect the susceptibility to infection by HIV. One well documented example of this is the 32 base pair deletion in the CCR5 co-receptor gene (CCR5Δ32). There is a decreased susceptibility to infection in individuals that are homozygous for this mutation (Huang et al., 1996). Disease progression may depend on the haplogroup of the CCR5 gene promoter region expressed (Nguyen et al., 2004). Mutation in other co-receptors such as CCR2 (Smith et al., 1997) or mutations in ligands for co-receptors (Hogan and Hammer, 2001) may also affect the rate at which disease occurs.

In 1986 it was reported (Walker et al., 1986) that $CD8^+$ T cells were able to block active HIV replication through non-cytolytic virus suppressive mechanisms. The soluble factors which suppress primary isolates of HIV were named collectively as 'CD8 antiviral factors' or CAF. Later, Cocchi and colleagues (Cocchi et al., 1995) reported that activated CD8 cells release ligands for chemokine receptors which have this suppressor activity. These ligands were called RANTES (regulated upon activation normal T-cells expressed and secreted), macrophage inflammatory proteins 1α and 1β (MIP-1α and MIP-1β). These chemokines are the cause of the suppression of R5 tropic strains of HIV. SDF-1 was later reported to be the biological ligand for CXCR4 and capable of preventing X4 tropic HIV cell entry (Bleul et al., 1996).

Both plasmacytoid dendritic cells (PDC) and natural killer (NK) cells have a considerable role in the innate cellular immune response. Decreased interferon-alpha (INF-α) production reflects the loss of PDC cells and this is associated with increased HIV RNA levels and progression to AIDS (Soumelis et al., 2001). Without prior sensitisation or MHC restriction, NK cells are able to kill virus infected cells. NK cells appear to have a protective role against HIV infection, as shown in a study of intravenous drug users (IDUs). Scott-Algara and colleagues (Scott-Algara et al., 2003) found that there was enhanced NK-cell function (production of the cytokines IFN-γ and TNF-α and the β chemokines CCL3, CCL4 and CCL5) in uninfected but exposed IDUs, in comparison to those IDUs who became HIV-1 infected. NK cells may also increase CC-chemokine production, thereby suppressing viremia (Kottilil et al., 2003).

The role of neutralising antibodies in acute infection is still debated. Neutralising antibodies are not detected until approximately 3-8 months following seroconversion (Figure 1.9) (Moog et al., 1997). It has been suggested that neutralising antibodies appear sequentially, at first against the autologous infecting HIV-1 variant and then against subsequent variants of the virus that emerge as a result of immune pressure; this causes a broadening of the neutralising antibody response (Moog et al., 1997; Wrin et al., 1994). Mutations in the Env protein that cause altered conformation or a change in the glycosylation pattern of the

envelope ("glycan shield"), leads to the emergence of neutralisation escape virus (Kwong et al., 2002; Wei et al., 2003). Long term non-progressors with established infection show broad and high titred neutralising activity compared to patients with rapid disease progression (Cecilia et al., 1999; Pilgrim et al., 1997).

Cytokines produced by $CD4^+$ T cells activate effector cells within the immune system (Gloster et al., 2004; Ostrowski et al., 2003). The types of cytokines produced determine whether a Th1 (T cell dominated) of Th2 (B-cell dominated) response results (Kedzierska and Crowe, 2001). A balance of these responses in generally considered to be required for an effective immune response to HIV (Lehner, 2003). Control of viremia depends on a strong proliferative response to HIV antigens and production of Th1 type cytokines as such responses inversely correlate with plasma virus load (Rosenberg et al., 1997). This has been confirmed in SIV and SHIV infected monkeys (McKay et al., 2003). Those patients who developed a strong $CD4^+$ T cell response to initial infection are generally those with slower disease progression whilst those with a reduced production of cytokines were more likely to be rapid progressors.

HIV-1 specific cytotoxic T lymphocytes (CTL) were reported in 1987 in HIV-1 seropositive individuals (Walker et al., 1987). These cells prevent HIV multiplication by killing infected cells. Several candidate vaccines have been shown to produce potent CTL responses which were partially protective against both pathogenic and non-pathogenic viral challenges in the non-human primate model (Allen et al., 2000; Barouch and Letvin, 2000; Egan et al., 2000; Hanke et al., 1999; Robinson et al., 1999). The earliest adaptive immune response in acute HIV-1 infection is the production of HIV-1 specific $CD8^+$ T-cells which is associated with the decline in viremia at this stage (Figure 1.9) (Borrow et al., 1994; Koup et al., 1994). Those individuals with a strong HIV-1 specific CTL response are likely to be those with slow progression to disease (Cao et al., 1995; Klein et al., 1995). However, HIV-1 infection is not prevented by a strong CTL response (Borrow et al., 1997; Goulder et al., 1997; Price et al., 1997), possibly indicating the need for a good pre-existing humoral (neutralising antibody) response as well. Many factors such as immunological pressure, fitness of virus for replication and HLA haplotype of the

individual contribute to the emergence of virus that escapes CTL and neutralising antibody control.

1.10.2 HIV-2

As for most aspects of HIV-2 infection the immune response to the virus is not as extensively studied as for HIV-1. The main reason for $CD4^+$ T cell depletion due to HIV infection is immune activation (Anderson, Ascher, and Sheppard, 1998; Cohen Stuart et al., 2000; Grossman et al., 2002; Hazenberg et al., 2000), which correlates strongly with disease progression (Giorgi, Hausner, and Hultin, 1999; Giorgi et al., 2002; Hazenberg et al., 2003). Many researchers state that the mechanisms of infection and response to HIV-2 are similar to HIV-1 and it has been proposed that there is a direct causal relationship between immune activation and $CD4^+$ T cell depletion in HIV-2 infection (Sousa et al., 2002). However, HIV-2 infection is associated with less rapid $CD4^+$ T cell depletion and a low level of viral replication compared to HIV-1 infection (Berry et al., 1998; Blaak et al., 2004; Jaffar et al., 1997; Popper et al., 2000; Shanmugam et al., 2000). This probably explains why those infected with HIV-2 have a longer asymptomatic period than those infected with HIV-1 (Jaffar et al., 1997; Marlink et al., 1994; Schim van der Loeff et al., 2002; Whittle et al., 1994). In turn this is probably due to stronger immune responses to HIV-2 than HIV-1, decreased T cell activation and down-regulation of CCR5 (Akimoto et al., 1998; Hanson, 2005; Shea et al., 2004).

Several studies by Kaneko and colleagues (Akimoto et al., 1998; Kaneko et al., 1997b; Neoh et al., 1997) have shown that HIV-2 can bind to the α-chain of $CD8^+$ T cells *in vitro* and this interaction activates phosphorylation of $p56^{lck}$. This interaction also occurs when HIV-1 binds $CD4^+$ T cells and it is known to help induce immune dysregulation, which includes T cell activation, CD4 down-modulation, T cell anergy (decreased production of interleukin-2) and apoptotic death of $CD4^+$ T cells (Banda et al., 1992; Kaneko et al., 1997a). Enhanced expression of various cytokines, including TNF-α and IL-6, occur as a result of the interaction. Production of these cytokines, mainly by macrophages, causes further T-cell abnormalities and B-cell dysfunction (Breen et al., 1990; Kaneko et al., 1997a).

The binding affinity of HIV-2 to $CD4^+$ or $CD8^+$ T cells is known to be lower than that of HIV-1 to $CD4^+$ T cells (Akimoto et al., 1998; Moore, 1990). This may be a factor in the slower spread and lower infectivity of HIV-2 compared with HIV-1. Another reason may be HIV-2's weaker signal transduction in $CD4^+$ cells which probably results in a reduced induction of envelope glycoprotein-induced cell abnormalities (described above). Akimoto (Akimoto et al., 1998) also observed that factors which inhibit HIV replication such as IFN-γ and IL-16 showed an increased production from HIV-2 stimulated PBMCs compared to HIV-1 stimulated PBMCs, whilst IL-4, known to promote HIV replication, was produced in smaller quantities from HIV-2 stimulated PBMCs (Sekigawa et al., 1998).

As mentioned in the previous section β chemokines such as RANTES, MIP-1α and MIP-1β produced from $CD8^+$ T cells are known to inhibit HIV-1 and HIV-2 infection *in vitro* (Blackbourn et al., 1997; Cocchi et al., 1995; Levy, Mackewicz, and Barker, 1996). It has been demonstrated that β-chemokine production by HIV-1-stimulated PBMC, $CD4^+$ and $CD8^+$ T cells was lower than that of HIV-2 stimulated cells (Akimoto et al., 1998).

Rates of HIV-2 replication are variable in asymptomatic patients which may be due to host genetics (Alabi et al., 2003; Berry et al., 1998). Particular HLA types (e.g. B35, B53 or B58) in HIV-2 infected patients are known to produce CTL responses that are able to recognize HIV-1 Gag proteins (Bertoletti et al., 1998; Dorrell et al., 2001). Therefore, HIV-2 infected patients may be more able to control a subsequent HIV-1 infection. Much controversial data has been reported as to whether infection with HIV-2 may protect against acquisition of HIV-1 (Aaby et al., 1997; Ariyoshi et al., 1997; Norrgren et al., 1999; Travers et al., 1995; Wiktor et al., 1999). A review of the data up to 2001 by Greenberg (Greenberg, 2001) concluded that there was no protective effect. However, there is a possibility that the course of HIV-1 disease is alleviated by immune responses induced by co-infection with HIV-2 (Alabi et al., 2003) though there is no *in vivo* proof of this. In dually infected patients HIV-1 appears to be the pathogenic virus and HIV-2 replication is suppressed by HIV-1 (Sarr et al., 1999).

Studies based in Guinea Bissau communities have shown that mortality rates of HIV-2 infected individuals are only two to five-fold higher than those of HIV-uninfected individuals (Norrgren et al., 1995; Poulsen et al., 1997; Ricard et al., 1994). This contrasts to HIV-1 infected individuals living in East Africa having approximately a 10-fold greater risk of death (Nunn et al., 1997). This may relate in part to the properties of the HIV-2 glycoprotein. Cavaleiro *et al.*, (Cavaleiro et al., 2000) presented data which showed that *in vitro* HIV-2 gp105 had greater inhibitory properties on TCR-mediated lymphoproliferative responses and the expression of co-stimulatory molecules on T cells than did HIV-1 or SIV envelope proteins. This may be beneficial to the host, as evidence indicates that persistent T cell activation plays a central role in HIV pathogenesis. Studies have shown that virus transmission from infected to uninfected cells occurs principally in sites of continual activation of the T lymphocytes (Cohen, Kinter, and Fauci, 1997; Grossman et al., 1999; Swingler et al., 1999) and boosts in viremia occur following immune stimulation or opportunistic infections (Ostrowski et al., 1998; Staprans et al., 1995). The ability of the HIV-2 envelope glycoprotein to inhibit (even if only partially) T cell activation may contribute to reducing the continuous stimulation of the immune system observed in HIV infection which is associated with immune dysregulation (Cavaleiro et al., 2000).

Of the cytokines produced by immune activation TNF-α, a cytokine which can be both detrimental and protective in HIV infection, was found to have an increased production when monocytes were stimulated by HIV-2 gp105 (Cavaleiro et al., 2000). TNF-α's harmful effect during HIV-1 infection arises from its ability to induce HIV-1 replication through the activation of NF-κB, a cellular transcription nuclear factor which is a strong inducer of HIV-1 LTR-mediated transcription (Osborn, Kunkel, and Nabel, 1989). Such LTR-mediated gene expression is not modulated by TNF-α in HIV-2 (Hannibal et al., 1993). Therefore only the beneficial effects of TNF-α are relevant in HIV-2 infection which are its ability to synergise with IFN-γ to kill infected cells and inhibit the production of infectious HIV (Wong et al., 1988), probably through stimulating β-chemokine production (Cohen, Kinter, and Fauci, 1997).

It is possible that the distinctive immunosuppressive properties of the HIV-2 glycoprotein could be beneficial rather than damaging to the patient. By interfering with the increased cellular activation that is characteristic of HIV infection, it may limit bursts of viral replication that occur.

1.11 HIV/SIV Glycoproteins

HIV/SIV glycoproteins project as 'spikes' from the virus surface (Figure 1.8), with each virion carrying 8-10 spikes (Zhu et al., 2003). They are critical to the virus as they initiate the infection process and have a number of actions that contribute to viral pathogenesis (see section 1.10)

1.11.1 HIV-1

gp160 is the product of the *env* gene which through host-cell mediated processing is cleaved into the surface unit (SU) gp120 and the transmembrane (TM) gp41 domains that remain non covalently associated (Figure 1.10) (Allan et al., 1985; Robey et al., 1985).

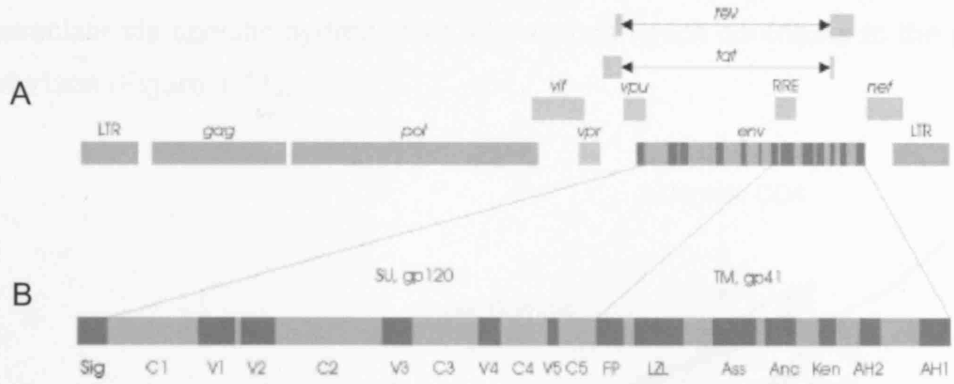


Figure 1.10: The *env*-gene and glycoproteins of HIV-1

The diagram is based on the genome of HIV-1_{HXBc2}. The location of the *env*-gene in the genome is shown together with the major overlapping viral elements (A). The linear features of the glycoprotein are shown below (B). The *env*-gene encodes the env proteins as a polyprotein sequence preceded by a signal sequence (Sig). The SU protein contains 5 regions with high sequence variability labelled V1 to V5. The remaining gp120 domains are referred to as constant regions C1 to C5. The transmembrane protein (TM, gp41) has an amino terminal fusion peptide (FP). Between the fusion peptide and the membrane anchor (Anc) lies the ectodomain containing the Leucine zipper like region (LZL) and the assembly domain (Ass). The cytoplasmic tail of HIV contains the Kennedy domain (Ken) and two amphipathic helices (AH2 and AH1).

The characteristic spikes observed on the surface of virions are trimeric combinations of three each gp120 and gp41 (Caffrey et al., 1998; Gelderblom et al., 1987; Gelderblom, Reupke, and Pauli, 1985; Lu, Blacklow, and Kim, 1995; Weissenhorn et al., 1997b). Exactly how and where gp120 and gp41 interact is still unknown, early speculation indicated a 'knob and socket' structure involving the disulphide bonded loop of gp41 (Schulz et al., 1992). However, more recent studies have indicated through mutational analysis that it is the hydrophobic groove of the gp120 core (Kwong et al., 1998) that is necessary for the interaction with gp41 (Figure 1.11) (Yang et al., 2003). This region includes portions of the C1 and C5 domains that have previously been implicated in the gp120-gp41 association (Helseth et al., 1991; Wyatt et al., 1997). Further to this mutational studies investigating the central ectodomain of gp41, revealed six tryptophan residues (positions 596, 610, 614, 623, 628 and 631), that are essential for the maintainence of the gp120-gp41 association (Figure 1.11) (Wang et al., 2002; York and Nunberg, 2004). Therefore, there is speculation that these two regions, the β-sandwich structure of the gp120 inner domain and the central ectodomain of gp41,

associate via specific hydrophobic interactions which contribute to the gp120-gp41 interface (Figure 1.11).

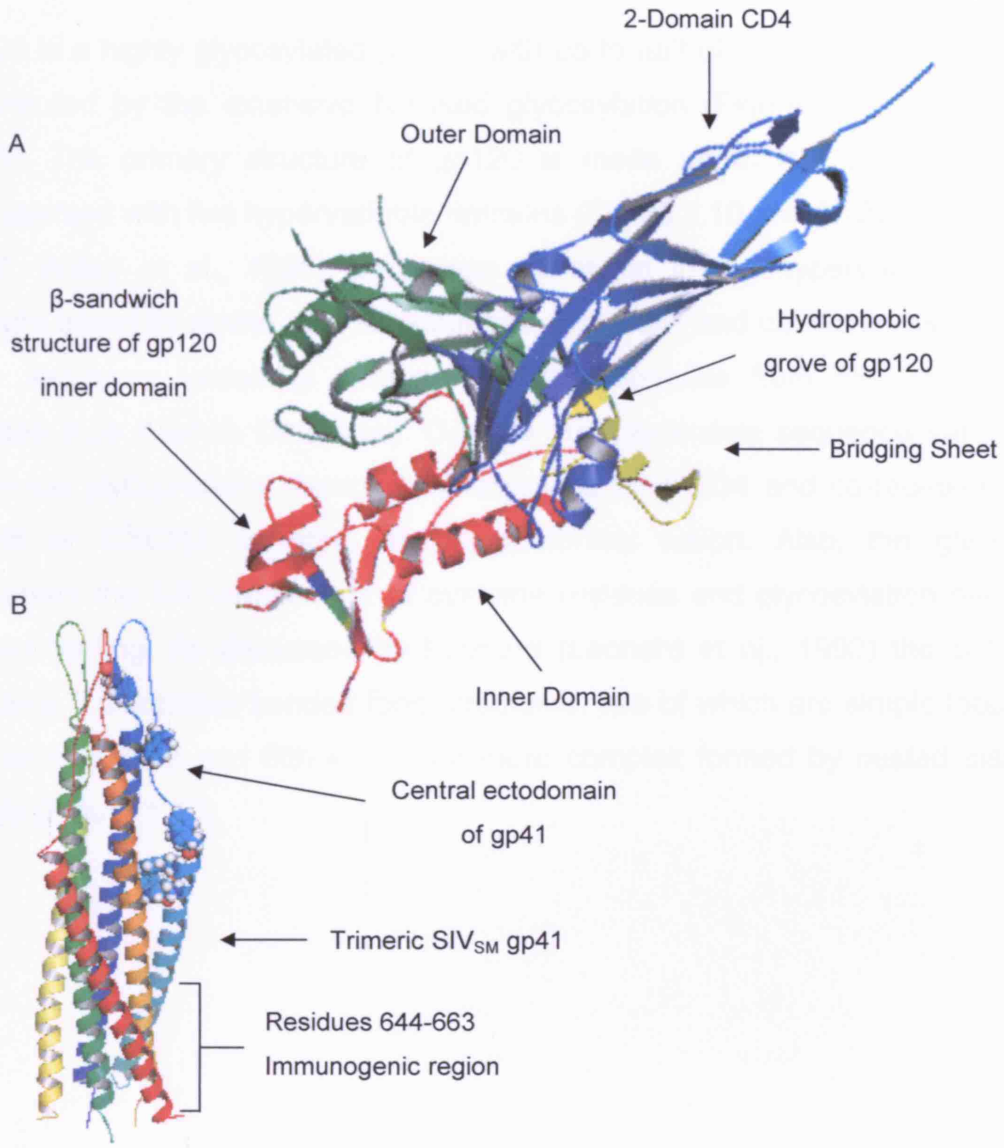


Figure 1.11: Structural Information regarding the gp120:gp41 interaction

The orientation of A and B would have the viral membrane near the bottom of the figure and the target cell membrane near the top. (A) The Cα tracing of the HXBc2 HIV-1 gp120 core (red, yellow and green, PDB No. 2B4C) and two-domain CD4 (blue) is shown, based on the 2.2-Å resolution structure (Kwong et al., 2000a). The gp120 core domains are depicted: inner domain (red), outer domain (yellow), and bridging sheet (green). The colour of the residue indicates the dominant phenotype associated with its alteration: yellow, wild-type; red, subunit dissociation; green, membrane fusion defects; and blue, processing defects. (B) Solution NMR structure of the post

six (SIV_{SM} does not have a tryptophan at position 614 – see text) tryptophan residues essential for maintenance of the gp120-gp41 association are shown by spheres (Wang et al., 2002; York and Nunberg, 2004). The spheres indicate the positions of these Trp residues on one subunit. (A was adapted from (Yang et al., 2003)).

gp120 is a highly glycosylated protein with up to half of its molecular weight being contributed by the extensive N-linked glycosylation (Figure 1.12) (Lasky et al., 1986). The primary structure of gp120 is made up of five constant domains interspersed with five hypervariable domains (Figure 1.10 and 1.12) (Modrow et al., 1987; Willey et al., 1986). Sequence variations in the hypervariable domains brought about by amino acid substitutions, insertions and deletions results in up to 25% sequence variability between gp120 molecules from the various HIV-1 isolates (Los Alamos Database). Despite such extensive sequence variation the envelope glycoproteins maintain the ability to bind CD4 and co-receptor, mainly CCR5 or CXCR4, to bring about membrane fusion. Also, the glycoprotein preserves the full complement of cysteine residues and glycosylation needed for correct folding. As discussed by Leonard (Leonard et al., 1990) the SU protein contains 5 disulphide bonded loop structures, two of which are simple loops while the second, third and fifth loops are more complex formed by nested disulphide bonds (Figure 1.12).

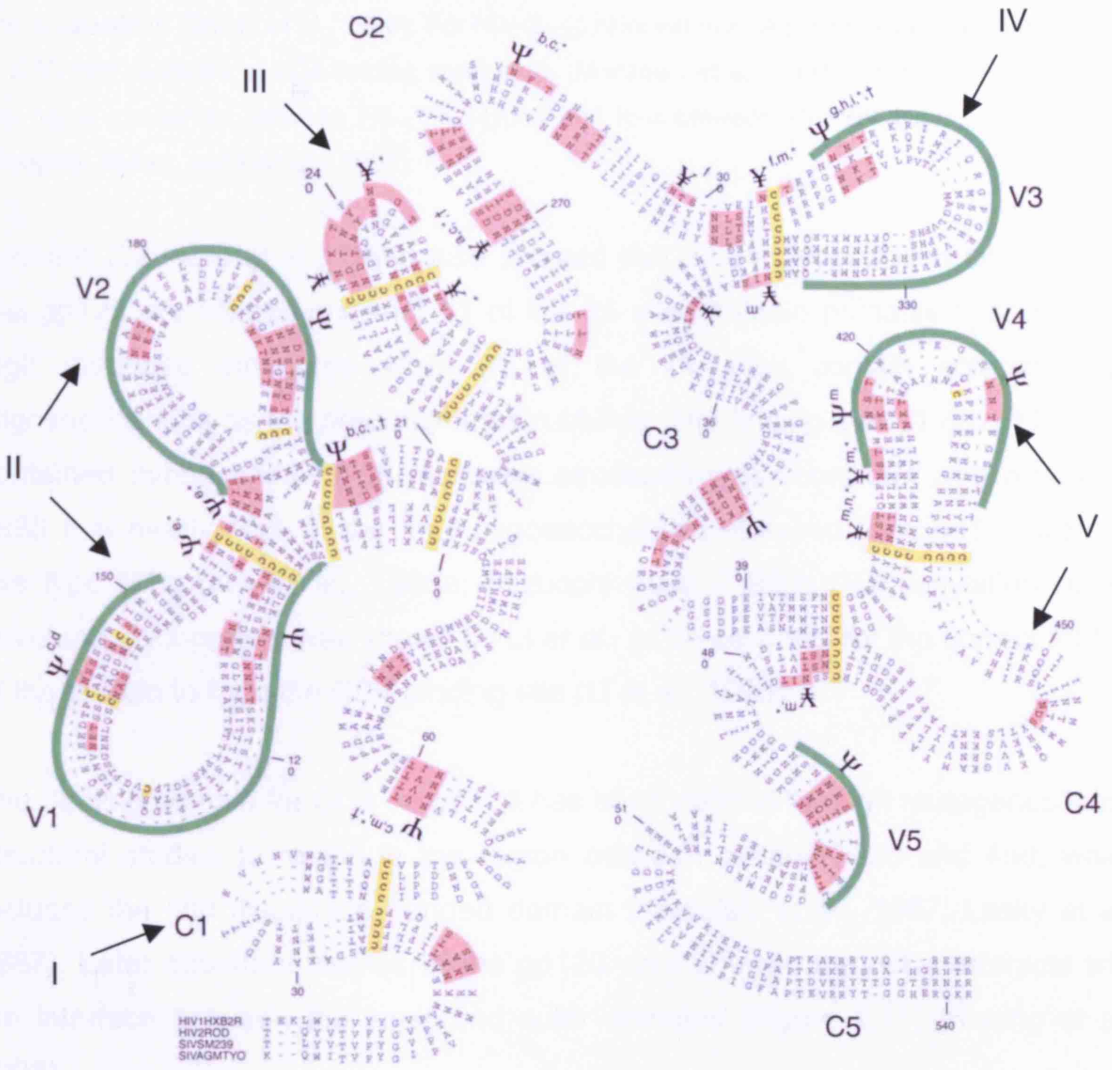


Figure 1.12: Summary of mutagenesis of HIV glycoprotein N-linked sequons

The glycosylation and disulphide bonding pattern of HIV-1 gp120 is adapted from (Leonard et al., 1990). Cysteine residues are shown in yellow and positions of constant (C) and variable (V) domains indicated. The limit of V-domains for HIV-1 are indicated in green. HIV-1_{HXB2R}, HIV-2_{ROD}, SIV_{SM239} and SIV_{AGMTYO} sequences were aligned using MULTAL and edited manually to align N-linked sequons (shown in red). The loops described by Leonard were designated I-V (Leonard et al., 1990). The complexity of oligosaccharides at specific sites for the HIV-1 glycoprotein are shown: complex-type (¥), high mannose and/or hybrid type (Ψ). Lower case letters adjacent to individual oligosaccharides refer to HIV-1 references that have shown removal of the sequon to: (i) be detrimental to infectivity/pathogenesis; a (Willey et al., 1988), b (Haggerty et al., 1991), c (Lee et al., 1992), d (Gram et al., 1994), e (Wu et al., 1995). (ii) Affect presentation of immunologic epitopes; f (Botarelli et al., 1991), g (Back et al., 1994), h (Yu et al., 1995), i (Schonning et al., 1996). (iii) Play a role in vertical transmission; j (Wolinsky et al., 1992), k (Orloff et al., 1993) l (Ahmad et al., 1995).

(iv) Affect the binding of a human monoclonal antibody that shows broad neutralisation of subtype B and A viruses;m (Trkola et al., 1996). For HIV-2_{ROD}, removal of a sequon has been shown to result in a 50-fold decrease in CD4-binding capacity; n (Morikawa et al., 1991). Thirteen of the sequons are highly conserved between HIV-1 subtypes and four between HIV/SIV types. Adapted from (Douglas, Munro, and Daniels, 1997).

Leonard (Leonard et al., 1990) also showed that all 24 glycosylation sites of HIV-1_{IIIB} gp120 are utilised and that 11 of the 24 sites contain primarily hybrid and/or high mannose structures while 13 of the 24 sites contain complex type oligosaccharides as the predominant structures. The finding that 11 out of 24 sites contained hybrid and/or high mannose structures was consistent with reports in 1988 that nearly 40% of the total oligosaccharides released from gp120 were of this type (Mizuochi et al., 1988a; Mizuochi et al., 1988b). Glycosylation of the envelope glycoprotein was shown by Li *et al.*, to be essential for the correct folding of the protein to form the CD4 binding site (Li et al., 1993).

The binding domain for CD4 on gp120 has been defined through mutagenesis and structural studies to reside in the region between residues 320 and 450, which includes the fifth disulphide bonded domain (Kowalski et al., 1987; Lasky et al., 1987). Later structural studies of the gp120 core showed that CD4 interacts with the interface between the inner and outer domains (Figure 1.11) (Kwong et al., 1998).

The structure of the core of gp120 in complex with the CD4 receptor and the Fab fragment from a neutralising human antibody was published in 1998 (Kwong et al., 1998). The HIV envelope glycoprotein has proved extremely difficult to crystallise due to the high degree of glycosylation and the many unstructured domains, represented by the surface variable loops. Consequently the currently crystallised state is of a severely truncated gp120 core which was stabilised by the Fab fragment and CD4 receptor. The primary sequence of this core is missing 52 and 19 amino acids from the N and C termini respectively and contains Gly-Ala-Gly tripeptide substitutions for V1/V2 (67 residues) and V3 (32 residues) loops. Over 90% of the carbohydrate was removed from the protein, using endoglycosidases D, F and H and glycopeptidase F, leaving only the linkages between the two core

N-acetyl-glucosamine residues. The structure revealed that a region called the bridging sheet links the two major domains (inner and the outer) of HIV-1_{HXBc2} gp120 (Figure 1.13) (Kwong et al., 1998) and this structure is highly conserved in the HIV-1_{YU2} gp120 core (Kwong et al., 2000a).

More recent work from the same group (Huang et al., 2005) has resulted in the crystal structure of a V3-containing HIV-1 gp120 core bound to the CD4 receptor and the Fab fragment of the X5 antibody (Figure 1.13). In this structure the V3 protrudes 30Å from the core such that the co-receptor binding tip of V3 is accessible. The availability of the V3 to the external environment would make it an easy target for antibodies and therefore may help explain its immunodominance (Huang et al., 2005). Neither of these structures is likely to represent the native virion bound structure of the protein as binding partners are known to alter the conformation of proteins (Thali et al., 1993), notably in the case of CD4 (Sattentau and Moore, 1991).

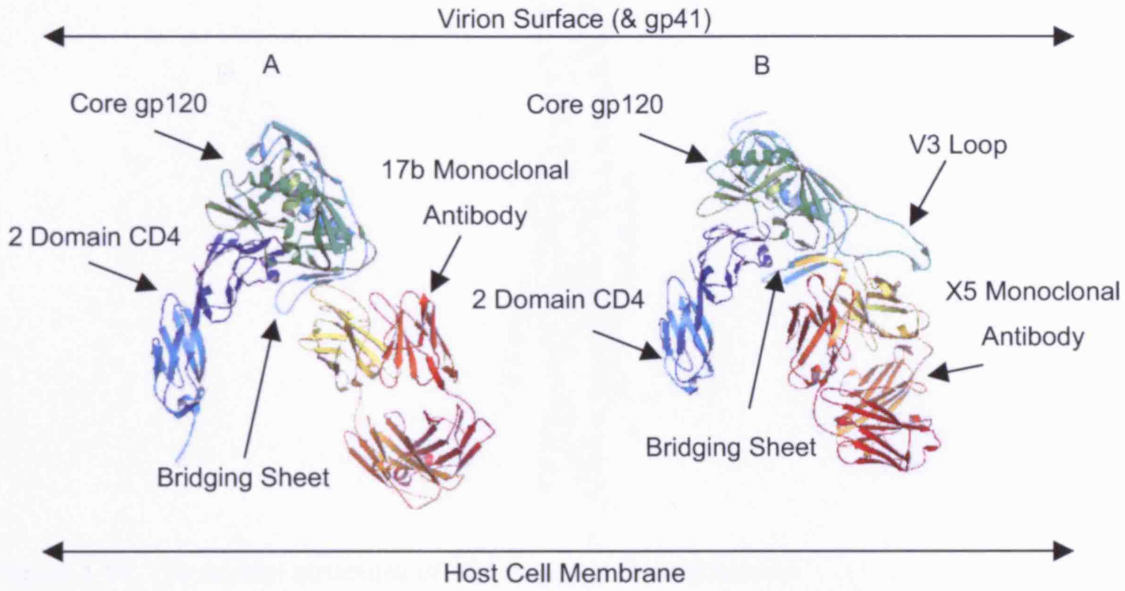


Figure 1.13: Comparison between the structures of HIV-1 gp120 core and HIV-1 gp120 core with V3

The crystal structures A (PDB No. 1GC1) and B (PDB No. 2B4C) are shown in the same orientation with the T cell membrane at the bottom of the figure and the gp41 binding domain (Figure 1.11) and hence the virus, at the top of the figure. The core gp120 (HIV-1 strains HXBc2 and JR-FL) (green and turquoise) with an intact V3 (indicated) is shown bound to the membrane-distal two domains of the CD4 receptor (light and dark blue) and the Fab portion of the 17B and X5 monoclonal antibodies (heavy chain is shown in yellow and the light chain is shown in orange). (Huang et al., 2005; Kwong et al., 1998).

Weissenhorn *et al.,* (Weissenhorn et al., 1997b) used X-ray crystallography to solve the structure of the protease resistant region of gp41 (Blacklow, Lu, and Kim, 1995; Lu, Blacklow, and Kim, 1995), which was made soluble with a GCN4 coiled coil in place of the amino-terminal fusion peptide (Weissenhorn et al., 1997a). The structure of the post-fusion form of the gp41 ectodomain was found to be an extended, triple stranded α-helical coiled coil with the amino-terminus at its tip (Figure 1.14). The amino- and carboxy- termini are found near each other at one end of the rod as the carboxy-terminal derived α-helix packs in the reverse direction on the outside of the amino-terminal derived coiled coil. These results concurred with those from two other groups who also crystallised smaller regions of gp41 (Chan et al., 1997; Tan et al., 1997).

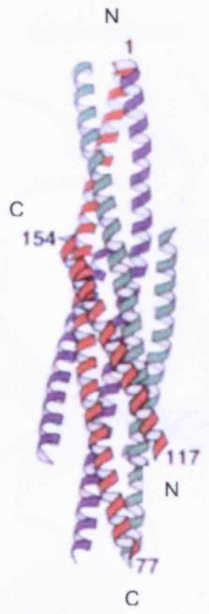


Figure 1.14: The crystal structure of HIV-1_{HXBc2} gp41 ectodomain

The amino (N) and Carboxy (C) termini of amino- ('N' peptide, residues 1-77) and carboxy- ('C' peptide, residues 117-154) terminal derived, protease resistant domains, or gp41 are shown. Adapted from (Weissenhorn et al., 1997b).

Although many groups have tried, as yet no structure has been published on the full ectodomain of the HIV-1 envelope glycoprotein in either a monomeric or, the generally accepted, virion bound trimeric state (Kwong et al., 2000b; Srivastava et al., 2002; Yang et al., 2000a; Yang et al., 2000b; Yang et al., 2002). Coiled coil domains such as GCN4, as used for crystallisation of the gp41 ectodomain, and the C-terminal domain of the Bacteriophage T4 protein Fibritin (Yang et al., 2002) (Figure 1.15), which is implicated in stabilising and initiating the formation of a coiled coil (Letarov et al., 1999; Strelkov et al., 1998; Tao et al., 1997), have been used to stabilise the HIV-1 glycoprotein ectodomain trimeric conformation in attempts to improve the probability of successful crystalisation trials.

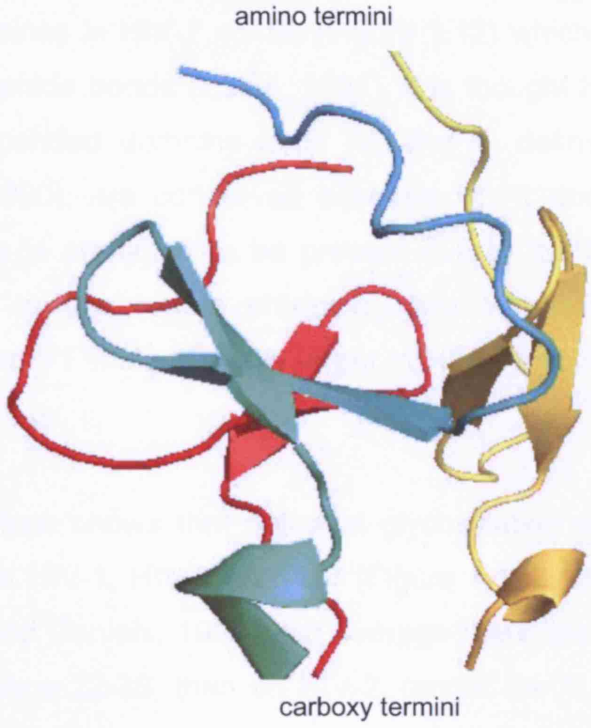


Figure 1.15: Structure of the trimeric C-terminal domain of Fibritin

The crystal structure of the trimeric C-terminal fibritin domain (GYIPEAPRDGQAYVRKDG-EWVLLSTFL adapted from PDB No. 1V1I) from the Bacteriophage T4 with the amino-termini at the top of the figure and the carboxy-termini towards the bottom of the figure. Adapted from (Papanikolopoulou et al., 2004).

1.11.2 HIV-2

As for HIV-1 the HIV-2 envelope glycoproteins are produced as a 140kDa precursor which is subsequently cleaved by host proteases into gp105 (SU) and gp36 (TM). The two subunits are non-covalently bound together and are generally thought to form trimers on the surface of the virion (Parekh et al., 1991), despite early evidence for the formation of homodimers (Rey et al., 1989; Rey et al., 1990). Functional studies have shown that HIV-2 gp105 binds the receptor CD4 but with a much weaker affinity than HIV-1, possibly by a factor of 25 (Moore, 1990). There is a lack of structural information on the HIV-2 envelope glycoprotein, with assumptions being made based on what we know about the HIV-1 and SIV envelope glycoproteins.

There are 22 cysteines in HIV-2 gp105 (Figure 1.12) which are proposed to form 11 intrachain disulphide bonds (Hoxie, 1991). It is thought likely that the positions of the disulphide-bonded domains I, III, IV, and V, defined by Leonard *et al.*, (Leonard et al., 1990), are conserved between HIV-1 and HIV-2. A finger like domain II structure is predicted to be present due to the two additional pairs of cysteine residues in this region producing two more disulphide bonds. The hypervariable region V1 is significantly larger in HIV-2 and SIV compared to HIV-1 (Figure 1.12).

Sequence comparison shows that potential glycosylation sites are generally well conserved between HIV-1, HIV-2 and SIV (Figure 1.12) (Chakrabarti et al., 1987; Douglas, Munro, and Daniels, 1997). On average there are more N-linked sugars found on HIV-1, range 22-38, than on HIV-2, range 24-30 (Douglas, Munro, and Daniels, 1997). There is a lack of characterisation of the types of glycosylation on the envelope protein, but, we do know that it is host-cell specific (Liedtke, Geyer, and Geyer, 1997).

Bour and colleagues have published several papers based on evidence for HIV-2 Env being able to carry out the same function as HIV-1 Vpu (Bour et al., 1996; Bour and Strebel, 1996; Bour et al., 1999). They propose that certain amino acids in the N terminal region of HIV-2 gp36 can enhance viral particle release but this is virus and host cell specific (Bour et al., 2003). These amino acids were defined recently by the confirmation of the importance of the alanine at position 598, a conserved Y-X-X-hydrophobic (YXXθ) motif in the membrane proximal part of the cytoplasmic tail and a less defined region in the ectodomain of gp36 (Abada, Noble, and Cannon, 2005).

A variety of constructs have been generated to allow production of HIV-2 envelope glycoprotein. Zhang *et al.*, (Zhang et al., 2001b) produced genetically stable gp105 of HIV-2_{ROD} in the methylotropic yeast *Pichia pastoris*. In 2002 the same group reported that they were able to express gp105 in yeast at an approximate concentration of 141mg/l, with an isoelectric point of pH 5.2 and the protein contained 35% carbohydrate (Zhang et al., 2002).

Kang *et al.*, (Kang, 1997) used the baculovirus expression system to express HIV genes. They expressed the surface glycoproteins of both HIV-1 and HIV-2, the majority of which were non-glycosylated as the signal sequences had been deleted to achieve high expression levels. Several groups have also produced part or all of HIV-2 or SIV_{MAC} gp140 using the vaccinia virus system (Benichou et al., 1992; Otteken, Voss, and Hunsmann, 1993; Spies and Compans, 1993). None of these reports contains details of glycoprotein purification of suitable quality and quantity for crystallisation trials.

More recently Sourial *et al.*, (Sourial et al., 2005) expressed a C- and N- terminal truncated gp105 with and without the hypervariable regions V1 and V2 of $HIV\text{-}2_{SBL\text{-}6669}$ in Chinese Hamster Ovary (CHO) *lec* 3.2.8.1 cells. Circular dichroism analysis indicated that there was secondary structure and that there was no appreciable difference between the two proteins. Surface plasmon resonance studies indicated that these HIV-2 constructs displayed native antigenic conformations as they bound HIV-2-positive sera, making them suitable for structural studies.

1.11.3 SIV

Recently an unliganded structure of the SIV_{MAC} gp120 core was published (Chen et al., 2005a; Chen et al., 2005b). The core construct was equivalent to that described for HIV-1 in 1998 (Kwong et al., 1998), but was produced in insect cells using a baculovirus expression system and retained its N-linked glycans. This complemented the available information and enabled a more thorough model of the conformational changes induced by CD4 binding to be proposed. The authors also suggested a model for the trimeric conformation of envelope glycoproteins on the virion surface unliganded bound to CD4 (Figure 1.16) (Chen et al., 2005b).

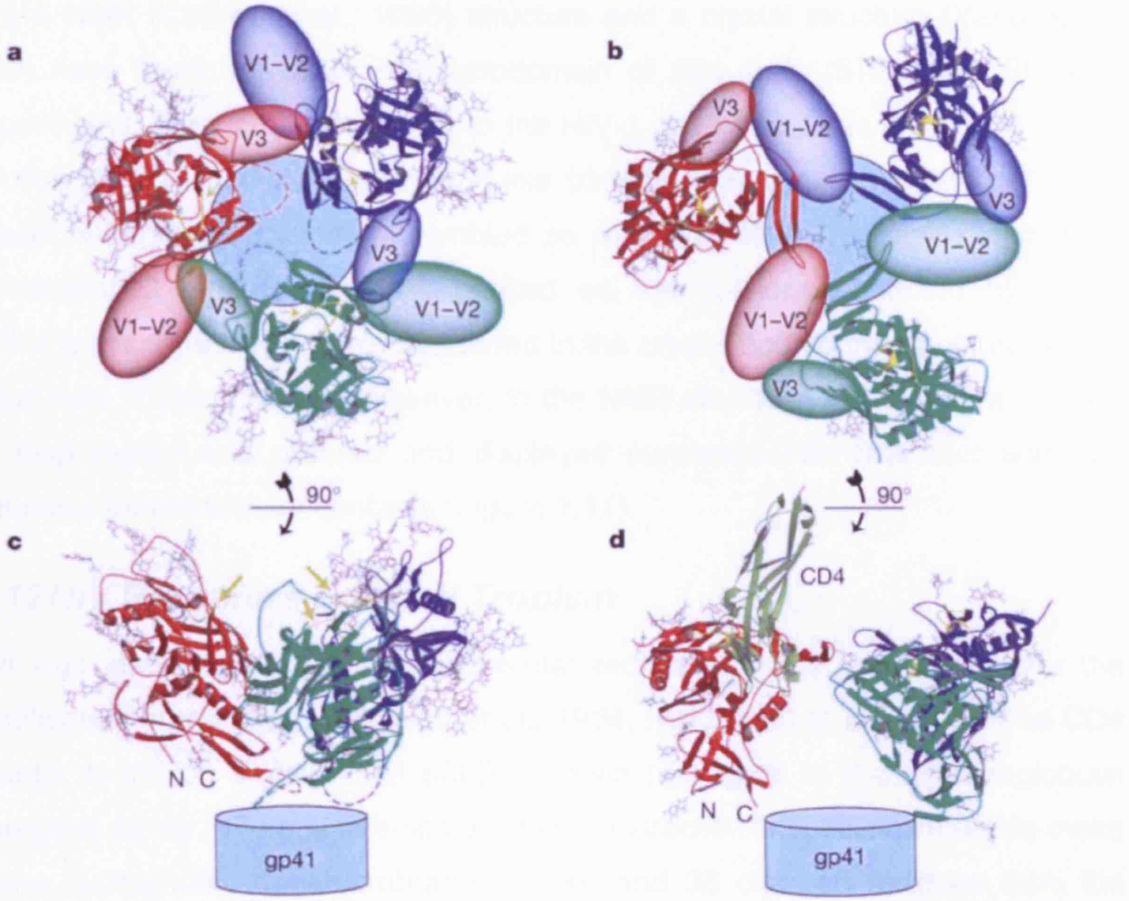


Figure 1.16: Proposed models for SIV gp120/gp41 trimers in unliganded and CD4-bound conformations

a: A trimer in the unliganded conformation, viewed along the three fold axis from outside the virion towards gp41. The polypeptide chain backbones are in ribbon representation; N-linked glycans are stick models; deleted V1-V2 and V3 segments are transparent balloons. The three monomers are in red, green and blue, respectively; the sugars, in grey. Gp 41 is shown as a light blue circle in the rear. b. The same view of a gp120/gp41 trimer as in a, but in the CD4 bound conformation, generated by superimposing the CD4-bound HIV gp120 structure onto the unliganded SIV gp120 subunits in panel a, assuming that the three-strand, inner-domain β-sheet remains roughly in place. Structural elements depicted as in a; CD4 omitted for clarity. c, 'side' view of the same model as in a. The N- and C- termini of the gp120 core are labelled; gp41 is shown as a light blue cylinder at the bottom. Green arrows indicate CD4 binding loops. d, Side view of the same model as in b. The first two domains of CD4 are shown in light green on only one gp120 monomer. N- and C- termini of the gp120 core are labelled. Adapted from (Chen et al., 2005b).

Both a NMR (Caffrey et al., 1998) structure and a crystal structure (Yang et al., 1999) have been solved for the ectodomain of SIV gp41 (SIV_{SM} and SIV_{MM239} respectively). They are both similar to the HIV-1 gp41 structures discussed above (Section 1.11.1) in that they are rod-like trimeric structures comprising of three parallel N-terminal α-helices assembled as a coiled coil in the centre with three anti-parallel C-terminal α-helices packed on the outside connected by highly flexible loops. The loops were disordered in the crystal and so the structure of that region was not determined. However, in the NMR structure, (Caffrey et al., 1998) the loop region was ordered and displayed numerous intermolecular and non sequential intramolecular contacts (Figure 1.11).

1.12 HIV Receptors and Cell Tropism

CD4 was identified as the specific cellular receptor for HIV very soon after the identification of the virus (Dalgleish et al., 1984; Klatzmann et al., 1984). The CD4 receptor found on T cells is a 58kDa protein belonging to the immunoglobulin supergene family, 370 amino acids are found extracellularly, 25 amino acids make up the hydrophobic transmembrane domain and 38 charged residues form the cytoplasmic domain. The extracellular region of CD4 has four N to C terminal arranged domains (D1-D4) (Clark et al., 1987; Maddon et al., 1985; Maddon et al., 1987; Sattentau, 1992) and crystal structures of D1 and D2 containing the site required for gp120 binding have confirmed that they share a basic structure comprising a stable fold of two β-pleated sheets composed of anti-parallel β-strands (Ryu et al., 1990; Wang et al., 1990). The cytoplasmic domain of CD4 is highly conserved among mammalian species, whereas the transmembrane and extracellular regions show only 55% homology between humans and mice (Littman, 1987). HIV-1 gp120 is unable to bind murine CD4 and sequence comparison between mouse and human CD4s was used to direct CD4 mutagenesis to determine what region of human CD4 were important for gp120 binding (Arthos et al., 1989). Residues on CD4 which are important for binding have been identified by many methods and amino acids 40-55 are critical for binding gp120 (Brodsky et al., 1990; Jameson et al., 1988; Mizukami et al., 1988; Peterson and Seed, 1988). This is also the site where CD4's natural ligand, MHC Class II molecules, bind (Clayton et al., 1989; Fleury et al., 1991; Houlgatte et al.,

1994). Other regions of the CD4 molecule required for gp120 binding encompass residues 81-94 (Kalyanaraman et al., 1990; Lifson et al., 1988).

The crystal structure of the CD4 bound gp120 core (Figure 1.13) provided the details on this interaction, showing it interacts at the interface of the inner and outer domains of gp120 (Kwong et al., 1998). CD4 makes extensive contacts over approximately 800Å^2 of gp120 surface, binding to a recessed pocket within the glycoprotein (Wyatt and Sodroski, 1998). A 10Å deep cavity occurs at the gp120-CD4 interface the opening of which is occupied by Phe^{43} of CD4, which by mutagenic analysis has been shown to be essential for CD4 binding (Arthos et al., 1989; Ashkenazi et al., 1990; Brodsky et al., 1990; Choe and Sodroski, 1992; Peterson and Seed, 1988; Ryu et al., 1990; Wu, Kwong, and Hendrickson, 1997). There is also a salt bridge, Asp^{368} of gp120 to Arg^{59} of CD4 and hydrogen bonds between the two proteins.

It soon became clear that additional factors must be involved in virus-host cell fusion allowing viral entry into cells, as some cells known to express CD4 were able to bind HIV without being infected (Broder et al., 1993; Chesebro et al., 1990; Clapham, Blanc, and Weiss, 1991; Dragic et al., 1992).

As mentioned previously the first indication of the importance of chemokines and their receptors in HIV pathogenesis was that HIV infection could be inhibited by soluble factors produced by CD8$^+$ T cells (Walker and Levy, 1989). This lead to the identification of these soluble factors now called β-chemokines, e.g RANTES, MIP-1α and MIP-1β (Cocchi et al., 1995). The β-chemokines were able to prevent HIV infection of transformed T cell lines (T-tropic virus strains) which enabled the identification of CXCR4 as a major co-receptor (Feng et al., 1996). Subsequently, the co-receptor responsible for HIV infection of primary macrophages (M-tropic virus strains) was identified as CCR5 (Alkhatib et al., 1996; Deng et al., 1996). Currently, nineteen of these seven-transmembrane domain G-protein coupled receptors (GPCRs) have been identified, *in vitro*, as co-receptors for HIV-1, HIV-2 and SIV (Table 1.5) (Simmons et al., 2000).

Receptor[a]	Ligands[a]	Predominant expression/Tissue distribution	Virus[b]	Ref
CCR1	MIP-1α, MIP-1β, RANTES, MCP-3	Monocytes, NK Cells, activated and naive T lymphocytes, immature DC	HIV-2, SIV	(Bron et al., 1997, Guillon et al., 1998, McKnight et al., 1998)
CCR2b	MCP-1, MCP-2, MCP-3, MCP-4	Monocytes, activated T lymphocytes, B cells	R5X4, HIV-2, SIV, HIV-1	(Bron et al., 1997, Chen et al., 1998, Doranz et al., 1996)
CCR3	Eotaxin, RANTES, MIP-1α, MCP-3, MCP-4	Th2 polarised T cells, eosinophils, basophils, activated T lymphocytes, microglial cells, astrocytes	R5X4, HIV-1 NSI	(Bron et al., 1997, Choe et al., 1996, Sol et al., 1997)
CCR4	TARC, MDC	Peripheral blood lymphocytes, thymocytes, B cells, NK cells, monocytes and neutrophils	HIV-2	(Bron et al., 1997, Guillon et al., 1998, McKnight et al., 1998)
CCR5	MIP-1α, MIP-1β, RANTES, MCP-2	IL-2 cultured activated T cells, monocytes, Th1 type T cells, memory T cells, immature DC, Langerhans cells, astrocytes, microglial cells	R5, R5X4, HIV-2, SIV	(Alkhatib et al., 1996, Bron et al., 1997, Deng et al., 1996, Dragic et al., 1996, Sol et al., 1997)
CCR8	I-309	Monocytes, thymocytes, Th2 type T cells, neutrophils, other tissues e.g. brain, lung, spleen, skeletal muscles	R5X4, HIV-2, SIV	(Rucker et al., 1997)
CCR9	TECK, RANTES, MIP-1α, MIP-1β	Thymocytes, lymphoid cell lines and PBMC	R5X4, HIV-1	(Choe et al., 1998)
CXCR2	GROα, GROβ, ENA-78, GCP-2, IL-8	Neutrophils, monocytes and a small portion of lymphocytes, placenta	HIV-2	(Bron et al., 1997)
CXCR4	SDF-1α, SDF-1β	$CD4^+$ and $CD8^+$ T cells, monocytes, DC, B lymphocytes, astrocytes, microglial cells, lung and spleen tissue	X4, R5X4, HIV-2	(Feng et al., 1996, Schols and De Clercq, 1998, Willett et al., 1997)
CXCR5	BLC/BCA-1	B lymphocytes, T lymphocytes type $CD4^+$, $CD8^+$, $CD25^-$, $CD44^+$, $CD62L^-$, $CD45RO^+$	HIV-2	(Kanbe et al., 1999)
CXCR6	CXCL16	$CD4^+$ T lymphocytes, memory T cells, NK cells, placenta, monocytes	R5X4, HIV-2, SIV	(Deng et al., 1997, Liao et al., 1997, Owen et al., 1998)
CX3CR1	Fractalkine	CD8+ T cells, NK cells, monocytes, neutrophils, brain, liver, skeletal muscle and peripheral blood	R5X4, HIV-2, SIV	(Reeves et al., 1997, Rucker et al., 1997)
GPR-1	ND	Tissue macrophages, brain	HIV-2, SIV	(Farzan et al., 1997, Shimizu et al., 1999)
GPR-15	ND	T cells, colon	R5X4, SIV	(Deng et al., 1997, Farzan et al., 1997)
Apj	ND	CNS	R5X4, SIV	(Choe et al., 1998, Edinger et al., 1998)
ChemR23	ND	Macrophages, DC	SIV, HIV-1	(Samson et al., 1998)
RDC1	ND	CD4+ T lymphocytes, CNS	HIV-2, SIV	(Shimizu et al., 2000)
BLTR	ND	Leucocytes, B lymphocytes	HIV-1	(Owman et al., 1998)
US28	RANTES, MIP-1α, MIP-1β	Fibroblasts infected with HCMV	R5X4	(Pleskoff, Treboute, and Alizon, 1998)

Table 1.5: Described co-receptors for HIV-1, HIV-2 and SIV

[a]The nomenclature adopted for chemokine receptors and chemokines were based on references (Murphy, 2002; Murphy et al., 2000). [b]Described viruses using this chemokine receptor for infection. Abbreviations: BCA, B-cell activating chemokine; BLC, B lymphocyte chemoattractant; CNS, Central Nervous System; DC, dendritic cells; ENA78, epithelial-derived neutrophil-activating peptide-78; GCP, granulocyte chemoattractant protein; GPR, G-protein coupled receptor; GRO, growth-related oncogene; HCMV, human cytomegalovirus; IL, interleukin; MCP, monocyte chemotactic protein; MDC, macrophage derived chemokine; MIP, macrophage inflammatory protein; ND, not determined; RANTES, regulated activation, normal T cell expressed and secreted;

SDF, stromal cell-derived factor; NK, natural killer; TARC, thymus and activation related chemokine; TECK, thymus-expressed chemokine. Reprinted with permission from Bentham Science Publishers Ltd (Azevedo-Pereira, Santos-Costa, and Moniz-Pereira, 2005).

Regardless of the number of co-receptors identified *in vitro* their *in vivo* use in HIV-1 infection is questionable, as the major co-receptors related to HIV-1 pathogenesis still appear to be CCR5 and CXCR4. Early in infection CCR5 (R5)-dependant strains of HIV-1 predominate. This is shown by M-tropic strains of HIV being important in transmission since people who are homozygous for the inactive Δ32CCR5 allele lack a functional CCR5 receptor and have a much reduced risk of infection with HIV (Dean et al., 1996; Liu et al., 1996; Mummidi et al., 1998; Samson et al., 1996). Following infection viral strains which can use CXCR4 as well (R5X4 strains) or rely on CXCR4 exclusively (X4 strains) become apparent in 40% of infected patients (Berger et al., 1998; Berger, Murphy, and Farber, 1999; Simmons et al., 1996). The appearance of these CXCR4 utilising strains is associated with a severe decrease in $CD4^+$ T cell count and enhanced disease progression (Bjorndal et al., 1997; Connor and Ho, 1994; Connor et al., 1997; Richman and Bozzette, 1994). Such clear CCR5 and/or CXCR4 targeting of HIV-2 strains is not present in light of the apparently wider range of chemokine receptors that can be used (Table 1.5) (Bron et al., 1997; Guillon et al., 1998; McKnight et al., 1998).

Successful viral entry may depend on the relative concentration of cellular receptors and their co-localisation in specific sites of the plasma membrane (Kozak et al., 1997; Kuhmann et al., 2000; Platt et al., 1998; Xiao et al., 1999). It is possible that increased concentrations of CD4 on the plasma membrane could compensate for decreased quantities of co-receptor such as CCR5 and vice versa (Platt et al., 1998). A study estimated that four to six CCR5 molecules were required to form a fusion pore (see later) using a mutant CCR5 with decreased affinity for HIV-1 envelope glycoproteins (Kuhmann et al., 2000).

There is some controversy as to whether G-coupled signalling initiated when the HIV SU binds to the co-receptor is required for efficient productive infection (Amara et al., 2003; Arthos et al., 2000). It has been proposed that the ability of HIV Env to mobilise calcium stores is required for replication in macrophages. The role that this CCR5 signal transduction might play is not fully understood but it may be required for post fusion events in viral replication (Arthos et al., 2000).

Determinants of co-receptor usage reside mainly in the hypervariable domain 3 (V3) of the SU Env glycoprotein (Cho et al., 1998; Hoffman and Doms, 1999; Hoffman et al., 1998; Hu et al., 2000; Smyth et al., 1998). However, changes in both V1/V2, through direct interaction with the V3 domain (Labrosse et al., 2001; Nabatov et al., 2004; Pollakis et al., 2001) and changes in glycosylation (Kolchinsky et al., 2001; Ly and Stamatatos, 2000; Malenbaum et al., 2000; Ogert et al., 2001; Pollakis et al., 2001; Wei et al., 2003) can alter which chemokine receptor is utilised for infection by HIV-1.

HIV-2 SU, like HIV-1, has five variable domains with five interspersed constant domains (Figures 1.10 and 1.12). Those residues which have been identified as essential for HIV-1 CD4 and co-receptor binding are well conserved in strains of HIV-2 and SIV (Kwong et al., 1998; Rizzuto et al., 1998). This implies that these areas might share a common structure and perhaps a similar function.

Although fewer HIV-2 strains have been as well characterised as HIV-1 strains, there appears to be less genetic heterogeneity between strains (Boeri et al., 1992; Damond et al., 2001) probably reflecting the lower rates of replication of HIV-2 within patients and reduced inter-patient transmission. What inter-strain variation exists for HIV-2 is more apparent in the V1/V2 domain than the V3 domain (Damond et al., 2001; McKnight et al., 1996) which contrasts with the situation in HIV-1. Hence for HIV-2, and possibly SIV, the V1/V2 domain (Almond et al., 1993) appears to be under strong selective pressure and may be the major determinant of co-receptor usage. However, studies have shown the C-terminal amino acids of the HIV-2 V3 loop to determine the co-receptor usage between CCR5 and CXCR4 (Isaka et al., 1999). Generally, HIV-2 Env V1/V2 domains are somewhat longer

than those seen in HIV-1 and contain four extra cysteine residues which will permit the formation of two additional intra-molecular disulphide bonds (Figure 1.12) (Douglas, Munro, and Daniels, 1997). The increased length might confer more inherent flexibility to the domain but this may be limited by the rigidity conferred by the additional disulphide bonds, though the latter may form in a number of ways. Whether flexibility or rigidity dominate, the V1/V2 domains of HIV-2 Env may represent a 'master key' able to 'unlock' many 'doors' as shown by the fact that gp105 can bind a wide range of chemokine receptors (Azevedo-Pereira, Santos-Costa, and Moniz-Pereira, 2005).

Additional to HIV-2's apparent promiscuous co-receptor usage it has shown a significant potential to become CD4-independent *in vitro* (Clapham, McKnight, and Weiss, 1992; Liu et al., 2000; Reeves et al., 1999; Willey et al., 2003). This is not the case for HIV-1 where CD4-independent strains are very rare (Dumonceaux et al., 1998; Hoffman et al., 1999; Kolchinsky et al., 1999; LaBranche et al., 1999). CD4-independent strains of HIV would be able to infect tissues of other body compartments such as the brain, testes, lymphoid tissue and lungs (Epstein et al., 1991; Gorry et al., 2002; Itescu et al., 1994; Sankale et al., 1996; Willey et al., 2003). However, CD4-independent strains of HIV-2 are much more susceptible to neutralisation than CD4-dependent strains. It is likely that epitopes, such as the co-receptor binding site, either hidden or not formed in the absence of CD4 engagement for a CD4-dependant strain, are exposed in a CD4-independent strain in order to allow infection (Hoffman et al., 1999; Kolchinsky, Kiprilov, and Sodroski, 2001). Such exposure would leave the SU open permanently to antibodies which are capable of inhibiting co-receptor binding.

HIV-2 has, through the use of a broad range of chemokine receptors (Azevedo-Pereira, Santos-Costa, and Moniz-Pereira, 2005), the ability to infect a wider range of human cells than HIV-1. Whilst there is a reported higher association with encephalitis in AIDS patients infected with HIV-2 than those infected with HIV-1, HIV-2 infection of a wider range of cells does not appear to occur (Lucas et al., 1993) notably in the brain (Morner et al., 2003).

1.13 Viral Fusion Mechanisms

Lipid enveloped viruses require the fusion of viral and host cell membranes for viral entry to occur. Two types of viral fusion protein have been identified which differ at the structural level but function similarly (Sollner, 2004). Type I fusion proteins are synthesised as large precursors, containing an abundance of α-helices, which form homotrimers and undergo proteolytic processing to yield metastable complexes that are anchored in the viral membrane by a hydrophobic domain near the C-terminus of the fusogenic component which carries fusion peptides at the N-termini of each monomeric unit. Since processing produces a metastable state that can be activated, it occurs late in the biosynthetic process. Type II fusion proteins are β-sheet rich, exist as dimers in their prefusion states, do not undergo proteolytic activation and have internal fusion loops rather than N-terminal fusion peptides.

HIV/SIV Env glycoproteins are examples of Type I fusion proteins. Following extensive work on its structure and function, and the application of modelling techniques, influenza haemagglutinin (HA) is the most highly characterised example of this type (Bentz and Mittal, 2003; Huang et al., 2003; Skehel and Wiley, 2000; Tamm, 2003). HA binds to sialic acid receptors on the surface of a host cell and the virus is subsequently taken up by endosome formation. The acidic environment of the endosome triggers a conformational change in HA causing the viral and endosome membranes to fuse, resulting in the release of the viral nucleocapsid into the cytoplasm of the cell. Whilst the membrane fusion mechanisms induced by HA and Env are likely to be similar, there are differences resulting from the properties of the proteins and the location at which membrane fusion occurs (reviewed in (Colman and Lawrence, 2003)). Notably, the subunits of HA monomers (HA_1 and HA_2) are stabilised by an inter-subunit disulphide bond whilst the HIV/SIV subunits (gp120/41 and gp105/36) rely on less-stable non-covalent interactions, and whereas HA membrane fusion takes place within endosomes, Env fusion occurs at the cell surface and is not dependent on low pH to trigger conformational change.

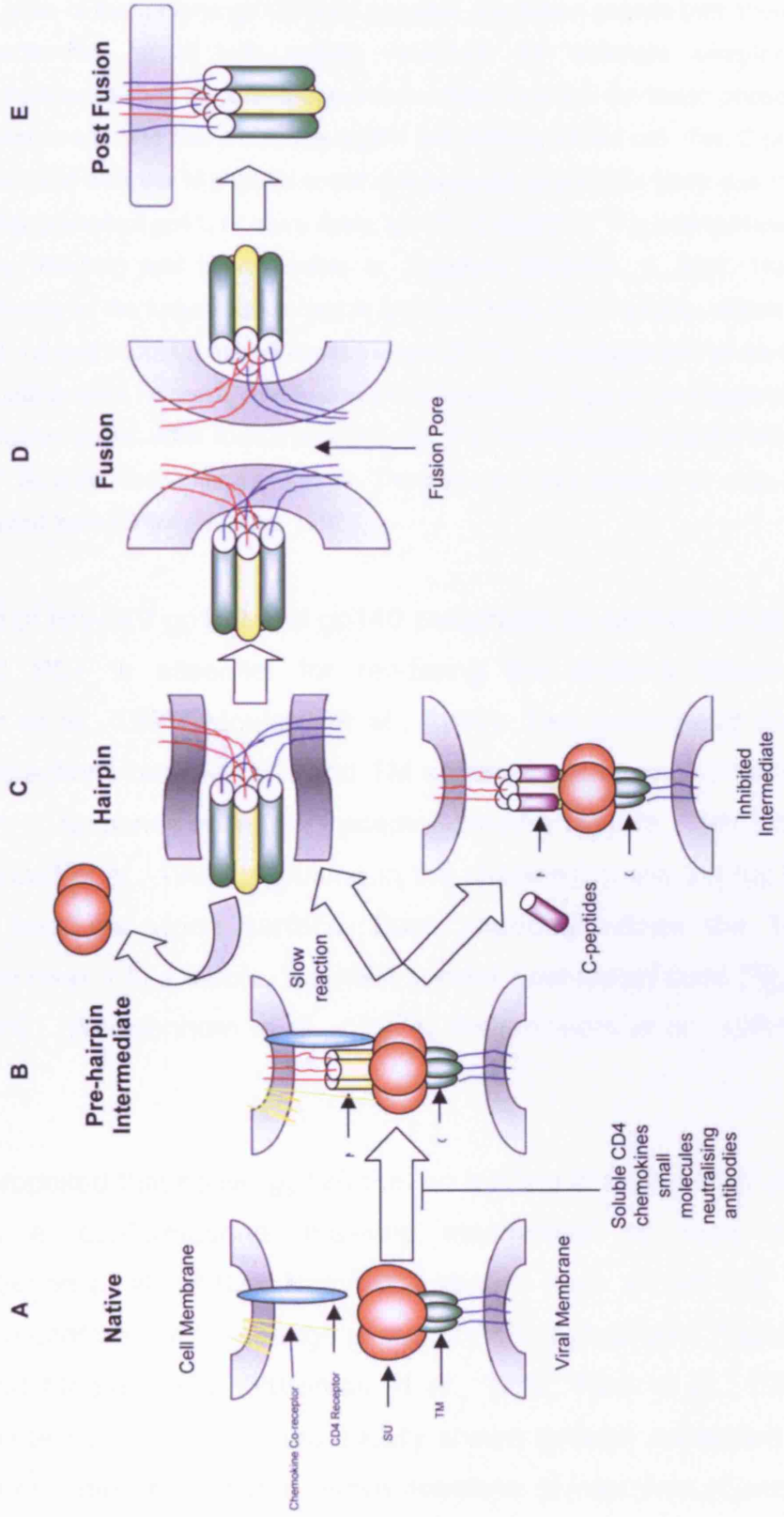

A
Native
Cell Membrane
Chemokine Co-receptor
CD4 Receptor
SU
TM
Viral Membrane
B
Pre-hairpin Intermediate
Soluble CD4
chemokines
small
molecules
neutralising
antibodies
Slow reaction
C
Hairpin
C-peptides
Inhibited Intermediate
D
Fusion
Fusion Pore
E
Post Fusion

Figure 1.17: Model of HIV Membrane Fusion

A: In the native state of the trimeric gp120/gp41 complex, the fusion peptide (not shown) is buried. B: Upon interaction of gp120 with cellular receptors, the envelope complex undergoes conformational changes to yield the pre-hairpin intermediate, in which the fusion peptide is inserted into the target membrane and the N-peptide region is a trimeric coiled coil. The C-peptide region has not yet associated with the N-peptide coiled coil because of a kinetic block due to association with either another portion of gp41, or more likely, gp120. C (bottom): This intermediate is relatively long-lived (many minutes) and is vulnerable to C-peptide inhibition. C (top): The pre-hairpin intermediate resolves to the fusion-active hairpin structure when the C-peptide region binds to the N-peptide coiled coil and adopts a helical conformation. D: This rearrangement results in membrane apposition. The interactions necessary for fusion are unknown, but may involve aggregation of gp41 trimers to form fusion pores. After fusion is completed, the fusion peptide and the transmembrane segment of gp41 lie within the same membrane. The steps at which various HIV entry inhibitors act are shown. Adapted from (Chan and Kim, 1998).

The cleavage of HIV/SIV gp160 and gp140 precursors by host-cell proteases such as furin and PC7 is essential for rendering the proteins fusion-competent (Hallenberger et al., 1997; Moulard et al., 1999). The consequent non-covalent stabilising interactions between SU and TM subunits are known to be labile being easily broken in response to either receptor binding (Moore, 1990) or antibody binding (Poignard et al., 1996), resulting in the shedding of the SU (gp120/gp105) components from the virion surface. Such shedding allows the TM subunit (gp41/gp36) to relax into a stable, 'six-helix bundle' post-fusion state (Figure 1.11B, 1.14 and 1.16E) (Weissenhorn et al., 1997a; Weissenhorn et al., 1997b; Yang et al., 1999).

It has been proposed that native gp120 (i.e. no binding to CD4 and/or co-receptor) may contain a 'conformational masking mechanism' to avoid neutralising antibodies (Kwong et al., 2002). Numerous studies have shown that on binding CD4 there is a conformational change in the HIV SU glycoprotein (Figure 1.16a-b) (Sattentau and Moore, 1991; Sattentau et al., 1993; Thali et al., 1993). These changes in protein architecture were initially shown through antibodies binding to CD4 induced epitopes, but not the native envelope glycoprotein (Gershoni et al., 1993; Thali et al., 1993; Wyatt et al., 1995). Later structural and functional studies have indicated the repositioning of up to 100 amino-acid residues in gp120 as

indicated by its characteristic slow association rate with CD4, changes in the circular dichroism spectrum, and large changes in thermodynamic parameters that are atypical of protein-protein complexes (Myszka et al., 2000). More recently, with the publication of the X-ray structure of the unliganded SIV gp120 core (Chen et al., 2005a; Chen et al., 2005b), a more detailed account of these conformational changes is available (Figure 1.17). It is widely believed that one of the results of conformational changes is the exposure/formation of the co-receptor binding site. From mutagenesis and binding studies the co-receptor binding site has been identified as several highly conserved residues within or surrounding the bridging sheet (Figure 1.13) (Basmaciogullari et al., 2002; Rizzuto et al., 1998). Part of this region, the V3 loop, has recently been crystallised in the context of the gp120 core bound to CD4 and the X5 antibody (Figure 1.13) (Huang et al., 2005). The structure shows the V3 loop projecting out towards the host cell membrane, hence making it available for co-receptor binding.

Possibly the most significant outcome of these conformational changes is the dissociation of gp120 from gp41 (Moore et al., 1990b). This may trigger the fusion activation of gp41 by releasing the steric constraints on it, thereby allowing the transition from a metastable conformation into the highly stable six-helix bundle (Caffrey et al., 1998; Chan et al., 1997; Tan et al., 1997; Weissenhorn et al., 1997b). In support of this, the regions in SU (C1 and C4/5) associated with TM interactions also alter their tertiary structure on CD4 binding (Moore and Sodroski, 1996). However, these epitopes only become exposed when gp120 dissociates from gp41 and this is dependant on co-receptor interaction (Finnegan et al., 2001). The precise timing of the dissociation of gp120 from its gp41 and CD4/co-receptor interactions during the fusion process is not known but conformational changes initiated by gp120 binding to soluble CD4 (sCD4) are not sufficient to activate gp41 (Si et al., 2004). Recent evidence shows that cell surface protein disulphide isomerase (PDI) can associate with CD4 resulting in PDI-CD4-gp120 complexes that allow PDI to reduce gp120 disulphide bonds (reviewed in (Ryser and Fluckiger, 2005)). Rupture of such structure-stabilising bonds may provide the trigger for greater conformational changes associated with gp120 removal from the fusion complex and activation of gp41.

The amino-terminus of TM produced by the cleavage of the precursor envelope glycoprotein contains a hydrophobic sequence termed the 'fusion peptide', as this is the sequence that becomes inserted in the host cell membrane. The C-terminal half of the TM protein contains the membrane anchor and a long cytoplasmic domain (Figure 1.10B). Between the fusion peptide and the membrane anchor is the 'ectodomain' of the TM containing sequences that were predicted to have helical secondary structure and to form coiled coils (Weissenhorn et al., 1996), as was shown by NMR and X-ray crystallography (Caffrey et al., 1998; Weissenhorn et al., 1997b). There are two regions of gp41 which are particularly immunogenic *in vivo* one spans residues, amino acids 598-604, that form the disulphide loop in the six-helix bundle whilst the other is located in the C-terminal α-helix encompassing residues 644-663 (Figure 1.11). The immuno-reactivity of these regions is substantially increased on sCD4 binding (Sattentau, Zolla-Pazner, and Poignard, 1995). This increase in immuno-reactivity could be due to a change in conformation of gp41 and/or gp120, which results in the exposure of these epitopes. The final gp41-based highly stable six-helix bundle that forms is characteristic of the post fusion conformation of the fusion protein (Figure 1.17E).

The most precise measurements to date of fusion kinetics have been obtained using dye transfer assays (Dimitrov, Golding, and Blumenthal, 1991; Kliger et al., 2001; Lineberger et al., 2002; Munoz-Barroso et al., 1998; Weiss et al., 1996). Studies of the fusion events have indicated that the binding of gp120 to CD4 receptor occurs after a time lag of 10-15 minutes following co-culture at 37°C (Dimitrov et al., 1992; Frey et al., 1995; Weiss et al., 1996). The binding and engagement of CD4 represents the first rate-limiting step in the membrane fusion process (Figure 1.17A). Following the lag phase there is a rapid rise in fusion yield that saturates at approximately 50-100min (Gallo, Puri, and Blumenthal, 2001).

Following CD4/co-receptor binding and the induced conformational changes, the gp41/36 fusion peptide is proposed to insert into the host cell membrane to form a 'pre-hairpin intermediate' (Figure 1.17B). Direct evidence for membrane insertion of fusion peptides in the context of whole fusion glycoproteins is limited (Harter et al., 1989; Nieva and Agirre, 2003). When CD4 and co-receptor associated beads were

added to cells expressing the HIV-1 envelope protein the membranes of cells expressing HIV-1 envelope glycoprotein were perturbed suggesting fusion peptide insertion (Dimitrov et al., 2001). More recently, utilising photosensitised labelling, direct measurements of fusion between HIV/SIV virions and appropriate target cells were made but, under the experimental conditions employed with such a sensitive technique, direct evidence for fusion peptide insertion was not obtained (Raviv et al., 2002). Despite this, earlier mutagenic analyses of the HIV fusion peptide in the context of the complete glycoprotein have shown support for the 'pre-hairpin' model (Cao et al., 1993; Delahunty et al., 1996; Felser, Klimkait, and Silver, 1989; Freed, Myers, and Risser, 1990; Kowalski et al., 1987; Schaal et al., 1995). Destabilisation of lipid bilayers provides a mechanism whereby the activation energy required for membrane fusion can be reduced. A common motif capable of causing such disruption, found in a diverse range of fusion systems, is the insertion of an α-helix at an oblique angle (Brasseur, 1991). Many studies have been conducted using synthetic peptides derived from the HIV fusion peptide and a range of synthetic/natural cell membrane targets (reviewed in (Nieva and Agirre, 2003)) and recent studies show/confirm that the fusion peptide can obliquely penetrate lipid membranes in an α-helical form when present at low concentration, though the form can change to an anti-parallel β-sheet when the peptide is either present at higher concentration or penetrating lipid bilayers of certain compositions (Castano and Desbat, 2005; Morris, Gao, and Wong, 2004; Sackett and Shai, 2005).

In the pre-hairpin intermediate, the N- and C-helices of the gp41/36 ectodomain are exposed prior to six-helix bundle formation (Figure 1.17B). Most HIV strains require interaction with CD4 and co-receptor to arrive at this stage though for some CD4-binding is sufficient (Furuta et al., 1998) whilst others, as indicated previously (section 1.12), have evolved to be dependent on co-receptor interaction only. Peptides based on the sequences of the N- and C-helices have been shown to block HIV entry into host cells (He et al., 2003). The peptide T-20, based on the C helix sequence, has been shown to bind the pre-hairpin intermediate of gp41 and block formation of the six-helix bundle (Furuta et al., 1998; Wild et al., 1992). T-20 related peptides are in clinical use as HIV entry inhibitors and it has been shown

that concentrations required to inhibit viral isolates can vary by two logs, dependent on gp120/co-receptor affinity, receptor density and fusion kinetics which presumably relate to the time spent in the transition from the pre-hairpin to hairpin states (Figure 1.17B-C; (Reeves et al., 2002)).

Efficient membrane fusion requires more than one trimer of Env to interact with CD4 and co-receptors. This highly cooperative process is affected by receptor density and Env-receptor affinity (Doms, 2000). It has been estimated that the formation of a fusion pore (Figure 1.17C-D) requires four to six CCR5 co-receptors (Kuhmann et al., 2000) and several CD4 receptors (Layne et al., 1990). It was proposed that clustering of receptors in domains such as lipid rafts in the plasma membrane of the host T cell might serve as targets for HIV Env mediated fusion (Dalgleish et al., 1984). Lipid rafts have been shown to be important for HIV-1 in terms of both viral entry and assembly of progeny virions (reviewed in (Campbell, Crowe, and Mak, 2001)). Whilst raft localisation of CD4 seems not to be essential for initial gp120 binding, post-binding fusion/entry steps (Figure 1.17B-E) may require lipid raft assembly for both virus infection (Popik and Alce, 2004) and cell-cell transfer of HIV (Jolly and Sattentau, 2005).

Very little is known about fusion pore formation and the actual fusion of the two membranes (Figure 1.17D). Models that have been used are based on the 'stalk-pore' paradigm (Chernomordik, Melikyan, and Chizmadzhev, 1987; Kozlov et al., 1989; Kozlovsky and Kozlov, 2002; Markin and Albanesi, 2002; Markin, Kozlov, and Borovjagin, 1984). According to this there are two stages of lipid rearrangements in fusion (Kozlov et al., 1989). The primary stage involves the distal (termed 'trans') membrane remaining intact whereas the contacting monolayer (denoted 'cis') merges with it. The intermediate structure, termed a stalk, becomes a hemi-fusion diaphragm, which ruptures allowing the formation of the complete fusion pore (Chernomordik, Melikyan, and Chizmadzhev, 1987). Transition of the hairpin structure to the post-fusion (Figure 1.17C-E) state is considered to supply the energy required for membrane fusion with the six-helix bundle structure (Weissenhorn et al., 1997b) representing the post-fusion state (Markosyan, Cohen, and Melikyan, 2003; Melikyan et al., 2000).

1.14 Role of the Envelope Glycoprotein in Pathogenesis

Both HIV-1 and HIV-2 envelope glycoproteins have a significant role to play in the pathogenesis of disease. During infection with either virus the envelope glycoproteins stimulate the immune system to produce both neutralising and non-neutralising antibodies. The latter can enhance infectivity *in vitro* a factor that may result in increased viral loads and consequently enhanced pathogenicity (Fust, 1997; Le Grand et al., 1991; Takeda and Ennis, 1990; Takeda et al., 1992).

Although neutralising antibodies can control viral loads for limited periods of time, the strong selective pressures they exert on the viral quasispecies results in rapid emergence/evolution of neutralisation-escape variants (Nakowitsch et al., 2005). The mechanisms for this have recently been elucidated and a multitude of factors are involved. As previously mentioned the amino acid sequence of HIV Env is highly variable, particularly within the solvent exposed surface loops of the glycoprotein (Gaschen et al., 2002), whilst the highly conserved regions, which could induce a broad neutralising antibody response, are either only transiently available (Chen et al., 2005b) or sterically hidden (Burton et al., 2004). Additionally HIV Env carries a dense variable glycan shield that can mask epitopes. (Wei et al., 2003). Finally, the mechanisim by which HIV Env - receptor binding occurs is characterised by a high level of intrinsic entropy that makes it a moving target for antibody neutralisation (Kwong et al., 2002).

Once infection has begun HIV-2 interacts with the immune system using mechanisms distinct from HIV-1, those that have been further elucidated are discussed below. HIV-1 and HIV-2 can bind $CD8^+$ T cells as well as $CD4^+$ T cells causing immune dysregulation (Akimoto et al., 1998; Kaneko et al., 1997b; Neoh et al., 1997; Saha et al., 2001) and up regulation of the expression of various cytokines (Breen et al., 1990; Kaneko et al., 1997a). One of these cytokines, TNFα, is capable of inducing virus replication (Osborn, Kunkel, and Nabel, 1989) but, through further interactions it kills infected cells, thereby inhibiting the production of infectious virions in HIV-1 infection (Wong et al., 1988). Such detrimental effects to

the host do not occur in HIV-2 infection as LTR-mediated gene expression is not under the control of TNFα in HIV-2 (Hannibal et al., 1993).

Compared to HIV-1 gp120, HIV-2 gp105 appears to somehow regulate pathogenesis as it is able to up regulate factors that inhibit replication (Akimoto et al., 1998) and down regulate factors that promote HIV replication (Sekigawa et al., 1998). This, linked with gp105's lower binding affinity for both CD4 and CD8 leads to a lower infectivity of HIV-2 compared with HIV-1 (Akimoto et al., 1998; Moore, 1990).

These and other studies lead us to believe that HIV-2 Env is capable of partially inhibiting T cell activation whereas HIV-1 Env is not (Cavaleiro et al., 2000). There is growing evidence to suggest that the continual immune activation characteristic of HIV-1 infection is important in the pathogenesis of the virus (Cohen, Kinter, and Fauci, 1997; Grossman et al., 1999; Swingler et al., 1999), resulting in shorter asymptomatic periods before onset of AIDS in HIV-1 infected patients compared to those infected with HIV-2.

1.15 Current Drug and Vaccine Approaches

Highly active antiretroviral therapy (HAART) currently uses the approach of several different types of drugs taken in combination to attack the virus at two or three stages in the retroviral life cycle (see section 1.7). The widespread use of these drugs has increased the life expectancy of those infected with HIV, prolonging the asymptomatic period of the disease (Gulick et al., 1997; Hammer et al., 1997). However, there are many challenges in managing the HIV chemotherapy regimes these include the development of resistance to classes of drugs, transmission of resistant viruses (Boden et al., 1999; Brenner et al., 2002; Little et al., 1999; Yerly et al., 1999) and treatment fatigue due to the development of serious drug associated pathology (Carr and Cooper, 2000; Carr et al., 1999; Safrin and Grunfeld, 1999). This provides the incentive for continued searching for novel therapies.

As mentioned previously, blocking of CD4 binding by use of sCD4 was not successful *in vivo* (Ashkenazi et al., 1991; Daar et al., 1990; Moore et al., 1993). A more attractive therapeutic agent to attack this stage of the lifecycle is Pro542, a recombinant fusion protein of both heavy and light chains of a human IgG2 antibody and the two amino terminal Ig domains of CD4 (Allaway et al., 1995). The interaction of this recombinant protein with Env is tetravalent, therefore it binds with a higher avidity than sCD4 (Zhu, Olson, and Roux, 2001). Pro542 has been shown to neutralise primary HIV-1 isolates *in vitro*, protect hu-PBL-SCID mice from virus challenge (Gauduin et al., 1996; Gauduin et al., 1998; Trkola et al., 1995), and reduce viral burden *in vivo* in humans (Jacobson et al., 2000; Shearer et al., 2000).

There are efforts to produce CCR5 binding inhibitors. These are small molecule inhibitors, such as TAK-779 and SCH-C, which inhibit replication of R5 strains when present at nanomolar concentrations (Baba et al., 1999; Dragic et al., 2000; Strizki et al., 2001). Such inhibitors have no effect against CXCR4-utilising strains.

It has been considered that long term inhibition of CXCR4 may have damaging consequences in humans as CXCR4 and its ligand SDF-1 are required in murine development (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). However, a low molecular weight compound, AMD3100, which blocks infection of CXCR4 strains has been discovered (De Clercq et al., 1994). Although this inhibitor was effective in mice (Datema et al., 1996), clinical trials were cut short due to a failure to meet efficacy goals and the occurrence of undesirable cardiac effects (Hendrix et al., 2004). Several other CXCR4 inhibitors are in development (Arakaki et al., 1999; Murakami et al., 1997; Nakashima et al., 1992; Tamamura et al., 1998) including ALX40-4 (Doranz et al., 1997) which has been well tolerated in phase I trials (Doranz et al., 2001). Despite such results, use of co-receptor inhibitors may be a dangerous strategy as they may drive the virus to utilise other co-receptors with unknown outcomes in respect of pathogenesis.

As well as T20 (see section 1.7) other peptides derived from the HR2 region of gp41 are being tested for their ability to inhibit HIV infection. T1249 is one such peptide, which appears likely to have some activity against HIV-2

(http://www.thebody.com/sfaf/spring00/entry.html, Spring 2000), its binding domain overlaps that of T20 however it is positioned to cover the highly conserved deep hydrophobic pocket within gp41 (Figure 1.18). Although both of these entry inhibitors exhibit a high degree of antiviral activity resistance has already been reported (LaBranche et al., 1999; Rimsky, Shugars, and Matthews, 1998).

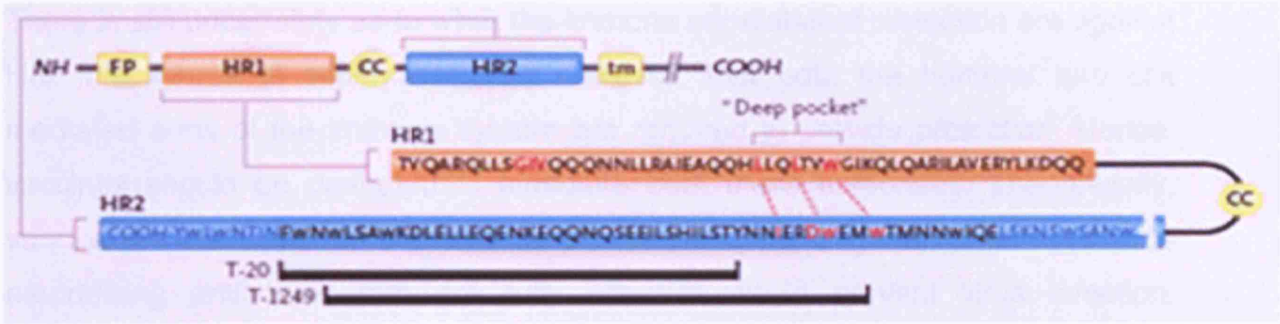


Figure 1.18: Schematic showing the gp41 amino acid sequence

Shown as a linear sequence, above, and with the relation between the HR1 and HR2 regions when gp41 is folded over into a hairpin configuration at a point where two cysteine residues [CC] form a disulphide-bonded loop, below. As shown at the bottom of the figure, T-20 and T-1249 are peptides derived from the HR2 segment. These peptides may act as competitive decoys for the process through which the extended coiled-coil structure of HR1 folds back to bind to corresponding regions of HR2 (with the T-1249 binding region extending farther along into the deep pocket sequence of HR1), thus disrupting the formation of the six-helix configuration required for membrane fusion. FP denotes fusion peptide, and tm membrane-spanning region. Adapted from (Kilby and Eron, 2003) with permission from The New England Journal of Medicine.

Other drugs in the pipeline include those that inhibit the retroviral enzyme integrase preventing strand transfer (Embrey et al., 2005; Hazuda et al., 2000). The most advanced of these is the diketo analogue S-1360 (Hazuda et al., 2000). It has shown strong antiviral activity *in vitro,* phase II trials were started in October 2002 and the launch of this drug is expected (Billich, 2003). A further novel target is the zinc fingers found in the nucleocapsid of HIV, which are involved in packaging the viral RNA into the budding virions. There are currently at least three zinc finger inhibitors under development (Saksena and Haddad, 2003).

New generations of protease and reverse transcriptase inhibitors are continually being developed. However, finding a novel drug which is not constrained under cross-resistance with other established drugs is proving more and more difficult. Therefore, the need for a vaccine is urgent and despite many efforts in this area of research there has been little success.

There is still uncertainty as to what the immune correlates of protection are against HIV infection. It is widely believed however, that both the humoral and cell mediated arms of the immune system are required to provide protection. Hence, vaccines should be designed to stimulate both these responses. Theoretically, vaccine pre-stimulation of the humoral part of the immune system to produce virus neutralising antibodies that are fully effective would prevent virus infection. However, the generation of an immunogen which can induce a broad enough neutralising antibody response, able to respond to heterologous viral challenge, has proved difficult.

Neutralising antibodies against a wide range of microorganisms target antigens present on their surfaces (Jiang, He, and Liu, 2005; Pantophlet and Burton, 2006; Xu et al., 2006). Human studies in the early 1990's showed that recombinant HIV Env protein was a poor immunogen *in vivo* (Belshe et al., 1993; Keefer et al., 1994; Salmon-Ceron et al., 1995; Valentine et al., 1996) and that even following seven immunisations of recombinant monomeric HIV-1 gp120, antibody responses were poor particularly in respect to broadly cross-neutralising antibodies (Gilbert et al., 2005a; Gilbert et al., 2005b). This poor response is generally attributed to the monomeric nature of gp120, its extensive glycosylation and the high degree of variation characteristic of the HIV Env glycoprotein (Moore and Burton, 2004). In attempts to overcome such poor responses and induce broadly neutralising antibodies, a move towards DNA vaccines (possibly with a protein boost) constructed of Env conjugated to adjuvanting-motifs such as murine C3d has occured (Bower et al., 2004; Li et al., 2006), but success has been limited.

Neutralising antibodies are present in the sera of almost every HIV infected individual, usually appearing after cell-mediated immunity. However, such antibodies are unable to control HIV replication and viral escape mutants readily emerge (Burton et al., 2004). The importance of neutralising antibodies in vaccine design for HIV was exemplified by the studies in rhesus macaques which showed that passive transfer experiments of pre-existing neutralising antibody can prevent HIV infection (Ferrantelli et al., 2004; Mascola et al., 2000; Veazey, Marx, and Lackner, 2003). HIV-1 and HIV-2 based vaccine studies in mice/rats and humans have yielded limited success in respect of raising neutralising antibodies (Bower et al., 2004; Matsushita et al., 1995; McKnight et al., 1996). However, the most promising broadly cross-neutralising antibodies to date have been derived from individuals infected with HIV-1.

Patients produce neutralising antibodies against different regions of HIV Env in response to HIV infection (Moog et al., 1997; Wrin et al., 1994). These include the V3 region (447-52D, the so called 'principle neutralising domain') (Gorny et al., 1992), the membrane proximal external region in gp41 (MAbs 2F5/4E10) (Buchacher et al., 1994), the CD4 binding site (MAb b12) (Burton et al., 1994), the co-receptor binding site (MAb 17b) (Robinson and Ho, Unpublished data; Thali et al., 1993) and the glycan rich region on gp120, defined by MAb 2G12 (Jeffs et al., 2002; Trkola et al., 1996). Although all of these regions have been characterised and responses have been observed in patients' sera, the majority of characterised neutralising antibodies are strain specific and directed against the V3 loop (Gorny et al., 2006). However, the virus can overcome such immune pressure due to its ability to tolerate amino acid substitution in the V3 loop and the belief that the V1/V2 loop (Pinter et al., 2004) and/or the carbohydrate moieties on the envelope (Wei et al., 2003) can mask it.

There are five well characterised HIV-1 specific broadly neutralising antibodies 17b, b12, 2G12 which recognise epitopes in gp120 and 2F5 and 4E10 which recognise epitopes in gp41 (Burton et al., 2004). Many groups are attempting to produce immunogens that will induce these types of antibodies (Belshe et al., 1998; Earl et al., 2001; Mascola et al., 1996; McGaughey et al., 2003; Srivastava et

al., 2003; Wrin et al., 1995). However, immunotherapy with combinations of such antibodies showed limited success highlighting the potential and limits of neutralising antibodies in controlling HIV-1 infection (Trkola et al., 2005). This might relate to an excess of high affinity neutralising antibody being required for virus neutralisation, as all the envelope glycoprotein SU domains need to be bound by antibody to prevent virus-cell fusion (Kim et al., 2001).

There have been two general approaches towards producing an Envelope based vaccine. The first of these utilises the belief that there are common neutralising antigenic structures/conformations, e.g. in the principle neutralising domain (V3 loop) (Javaherian et al., 1990; Javaherian et al., 1989). A recent example of this was highlighted by Gorny and colleagues where they identified the GPGQ motif, which is present in the V3 loop of over 85% of primary isolates of HIV-1, as a more potent immunogen than the GPGR motif mainly found in primary isolates of subtype B HIV-1 (Gorny et al., 2006). In addition, recent studies have shown that trimeric HIV-1 gp140 provided improved breadth of neutralisation compared with monomeric HIV-1 gp120 (Grundner et al., 2005; Yang, Wyatt, and Sodroski, 2001) and that the use of different adjuvants can enhance the response to heterologous virus challenge (Li et al., 2006).

The second approach is to use polyvalent sequence specific antigens as vaccines (Hurwitz et al., 2005). Investigations of this approach have shown that immunising with three subtypes of HIV-1 Env provided an increased titre and breadth of neutralising antibody response to a wide range of subtypes compared with immunising with one subtype of HIV-1 Env (Wang et al., 2006). However, immunisation with eight subtypes of HIV-1 Env at once provided no additional advantage. For both of these approaches it was observed that a prime of DNA, followed by a boost of recombinant protein, yielded optimal responses (Bower et al., 2006; Wang et al., 2006).

The Env glycan shield (Kwong et al., 2002; Wei et al., 2003) has also been investigated as the epitope of the well characterised neutralising antibody 2G12, is composed of a particular glycan array on HIV-1 Env (Calarese et al., 2005; Calarese et al., 2003; Sanders et al., 2002; Scanlan et al., 2002). Researchers have attempted to use clusters of mannose 9 glycans, conjugated to a strong T-helper epitope, as immunogens. Unfortunately, these proved to be poor immunogens as the majority of the IgG type antibodies were raised against the linkers in the conjugates (Ni et al., 2006). Conversely, removal of a number of the N-linked glycan motifs has been used in attempts to increase the sensitivity of the HIV protein to neutralisation. Mutation of four asparagines in the C3 region of the HIV-1 Env yielded an immunogen that induced, in rabbits, a higher and broader neutralising response than the fully glycosylated immunogen (Reynard et al., 2006).

In relation to HIV-2 Env fewer immunisation studies have been carried out and these have been conducted in rodents and non-human primates (Andersson et al., 1996; Locher et al., 2002; Locher et al., 2004). Generally such studies are following those performed with HIV-1 Env and further optimisation for both is required.

Clearly, design of vaccines targeting HIV-1/2 Envs is challenging and progress has been slow. All aspects of the vaccination protocol, from the design of the construct, type of priming (e.g. DNA/protein), number of booster innoculations required, route of administration, and monitoring of outcomes needs to be optimised. However, the literature suggests that the ideal vaccine candidate would incorporate common HIV Env immunogenic epitopes, expressed in a native trimeric conformation and administered using a DNA prime, recombinant protein boost strategy. The latter regime has shown promise in inducing both humoral and cellular immune responses (Shinoda et al., 2006).

Several dozen HIV candidate vaccines have entered phase I clinical trials in the last 15 years and many of these have focused on inducing cell-mediated immune response with particular focus on generating HIV-specific $CD8^+$ CTLs. Some of these have undergone, or are undergoing, phase II evaluation. However, as of July

2005, only two HIV vaccines have completed phase III trials and these were unsuccessful (http://www.cdc.gov/hiv/vaccine/vudev.htm Last Updated: September 09, 2005). Numerous vaccine concepts, schedules of immunisation, routes of administration, and adjuvants have been tested. However, there are severe limitations on using the classical methods of live attenuated HIV or whole inactivated HIV as a vaccine (Chertova et al., 2002; Whitney and Ruprecht, 2004). Hence, novel approaches are being employed.

Tat subunit vaccines have been explored as both humoral and cellular responses to this viral protein correlate with a delayed progression to disease in both monkeys and humans (Fanales-Belasio et al., 2002). However, as for other viral protein efficacy trials, only partial or mediocre immune responses were observed.

Monolipid tail lipopeptides may represent a promising avenue to follow, as synthetic fragments of HIV proteins (mainly Nef and Gag peptides) associated with lipids can induce broad cellular immune responses (Gahery et al., 2005; Gahery-Segard et al., 2003; Hosmalin et al., 2001).

Live recombinant vector vaccines use either a live attenuated viral or bacterial strain as a vector to carry HIV genes. These vaccines should be able to stimulate both arms of the immune response. Several different vectors have been investigated including Pox vectors (vaccinia, canarypox, fowlpox), Human adenovirus types 4, 5 and 7, defective alphavirus 'replicons' (e.g. Venezuelan Equine Encephalitis, Sinbis virus and Semliki Forest virus) and adeno associated virus. Such approaches have been reviewed in Voltan & Robert-Guroff 2003 (Voltan and Robert-Guroff, 2003).

Another novel concept has been the injection (into the epidermis) of naked purified plasmid DNA, carrying a gene for an antigen, which is under the control of a mammalian promoter leading to expression *in situ* and the triggering of a Th1 type immune response (Giri, Ugen, and Weiner, 2004). Prime-boost strategies, using nucleic acid vaccine and viral vector or subunit/peptide vaccine, are also being

investigated as they have been shown to induce stronger immune responses compared to vaccination with either vaccine alone (Excler and Plotkin, 1997).

Substantial evidence has shown that both the humoral and cellular immune responses are critical for controlling HIV infection and replication (Amara et al., 2001; Barouch et al., 2000; Igarashi et al., 1999; Shibata et al., 1999). It is believed that a strong anti-viral CTL response and production of neutralising antibodies can be achieved through vaccination with virus-like particles (VLP's). The minimum these VLP's have to contain is Gag and Env (Yao et al., 2003) however other proteins which are known to stimulate the immune response can be included (Akahata, Yang, and Nabel, 2005; Wyatt et al., 2004).

The enormous scientific challenge of developing a vaccine against HIV, a highly heterogeneous virus, is made more difficult by inadequate resources, clinical trial and regulatory capacity concerns and intellectual property issues. Currently (WHO, 2005), there are seventeen candidate vaccines in phase I trials, four in phase I/II and only one in phase III trial.

1.16 Why is Structural Information on HIV Envelope the Holy Grail?

As explained in previous sections the HIV envelope protein orchestrates the fusion of viral and cellular membranes during virus entry. This is a dynamic process which involves the sequential engagement of several different cellular receptors and attachment factors that function together to activate the fusion potential of Env. To protect this vital function HIV envelope glycoproteins have evolved to be inefficient at eliciting effective antiviral antibody responses.

The limited structural information of Env currently available has already led to the development of binding inhibitors, fusion inhibitors and co-receptor antagonists (see above). The fusion inhibitor T-20, is currently being utilised in HAART regimes to reduce viral load. However, studies have shown that the sensitivity of the virus to these drugs is strain dependent and the selection pressure exerted leads to escape virus.

Further structural information is required on the conserved HIV SU neutralisation epitopes which would facilitate the modification of this important antigen and allow the rational testing of hypotheses regarding its poor immunogenic properties. This would complement efforts to improve antigen presentation to the immune system and to create suitable animal models for the screening of vaccine candidates.

It has been shown that the HIV-1 envelope protein is responsible for lymphocyte anergy and apoptosis, as well as cytokine disturbances. This led to the hypothesis that the envelope protein itself has a role in the immunodeficiency associated with HIV-1 infection (Banda et al., 1992; Chirmule et al., 1990; Chirmule and Pahwa, 1996; Cicala et al., 1999; Schols and De Clercq, 1996). Generally HIV-2 is considered to be less pathogenic than HIV-1 and differences in the structure of HIV-1 gp120 and HIV-2 gp105 may contribute to the distinct pathogenicity of the two virus types.

Detailed knowledge of molecular structure of drug and vaccine targets aids design of therapeutic agents (Sirois, Sing, and Chou, 2005; Wang, 2003). Vaccine studies performed to date suggest that the native trimer of HIV Env as presented on the virion is the most effective immunogen of all the Env constructs designed as it produces an immune response which is active against heterologous virus challenge (Grundner et al., 2005; Yang, Wyatt, and Sodroski, 2001).

Although, the amino acid sequences of HIV-1 and HIV-2 Envs are only approximately 40% homologous they display a number of constant structural features such as the location of cysteine residues, certain N-linked glycosylation sequons and arrangement of protein domains (Douglas, Munro, and Daniels, 1997). This presumably relates to their mutual functions relating to receptor/co-receptor binding and membrane fusion (Azevedo-Pereira, Santos-Costa, and Moniz-Pereira, 2005; Moore, Trkola, and Dragic, 1997). Together, these observations suggest that the conformations of HIV-1 and HIV-2 Env are similar as is the case for Influenza haemagglutinins (HA) (Russell et al., 2004). There are 16 different influenza A subtype haemagglutinins and the crystal structures of representatives of a number of these are known. Despite the different HAs showing

approximately 50% amino acid homology only, their structures are superimposable reflecting their mutual functionality (Russell et al., 2006). Indeed, before the monomeric liganded structure of HIV-1 Env was solved in 1998 HA was used as a model for HIV-1 Env.

Only via a more thorough understanding of the native structures of the HIV glycoproteins, will we gain a better understanding of the mechanisms underlying their function and complex interaction with the host immune system. Possession of better structural information should assist in the development of more efficient drug and vaccine formulations to target HIV Env functions.

1.17 Objectives

It has been proposed that HIV-2 gp140 is more stable than HIV-1 gp160 such that it may provide a more stable structure for analysis and ultimately for crystallisation (Sattentau et al., 1993). During the course of molecular epidemiology studies our laboratory has obtained samples from a cohort of HIV-2 infected patients living in Caio, Guinea Bissau (Figure 1.4). Expression competent *env*-genes have been rescued from these samples (Daniels et al., unpublished). The aims of this PhD were therefore;

1. To modify, clone and express soluble rgp120 constructs of the HIV-2 glycoprotein either from the patient samples or from the original HIV-2 laboratory isolate HIV-2_{ROD}.
2. To develop a purification protocol for such rgp120s.
3. To assess the functionality and stability of the constructs generated as a measure of their suitability for structural studies.
4. Apply a range of techniques, e.g. Circular dichroism, analytical ultracentrifugation and electron microscopy to probe the structure of the rgp120.
5. To generate crystals for X-ray diffraction analysis to allow high resolution structure determination.

By expressing an ectodomain construct composed of SU and part of TM (gp120) I aim to add to the functional and structural knowledge of HIV glycoproteins in their native state. Additionally, highly purified rgp120 could be employed as a vaccine candidate and used to facilitate production of serologic reagents. Molecular structure determination and greater functional knowledge of purified HIV-2 envelope glycoprotein would broaden our understanding of HIV in numerous areas for example viral pathogenesis, viral tropism, viral fusion mechanisms. Such knowledge may facilitate the development of drugs to target the functions of HIV glycoprotein and enhance vaccine development by revealing antigenic structures.

It was recognised that this undertaking posed significant practical challenges that would need to be overcome for the project to be ultimately successful. These will be identified and resolved/discussed in the following chapters.

Chapter 2

Materials and Methods

2 Methods and Materials

2.1 Env-gene Templates & Construct Generation

2.1.1 Patient Samples

For samples collected from 22 different HIV-2 infected patients in Caio, Guinea Bissau in 1991, a total of 66 expression competent *env*-genes (Daniels et al., unpublished) were rescued into the pQ7 – transient expression cloning system (Douglas et al., 1996) (Appendix Table 1). Of these a single clone was selected for each of 6 patients to carry forward into constitutive expression studies to allow production of gp120s for structural analyses. Clones were selected based on their positions in a nucleotide-based phylogenetic tree (Appendix Figure 1) and the maintenance of a correct complement of cysteine residues to ensure correct protein folding. The glycoprotein sequences of the 6 clones selected are shown (Appendix Figure 2).

2.1.2 HIV-2_{ROD}

The full length infectious molecular clone pROD10 was obtained from CFAR, catalogue number EVA232pROD10 (Clavel et al., 1986b; Guyader et al., 1987). This was the complete proviral sequence of HIV-2_{ROD} constructed from λROD27, λROD35 and pSPE2. This clone served as a template for PCR-rescue of *env*-gene fragments for direct cloning into vector pEE14tPA2DCD4 (Figure 2.2).

2.1.3 Constructs

Four gp120 constructs were designed for each of the patient derived clones, with and without processing site mutations and with and without the fibritin domain (Figure 2.1). Yang *et al.*, (Yang et al., 2002) have added a fibritin domain to HIV-1 gp140 and shown it to make gp140 more stable to heat and reducing conditions. It was hoped that if bound to HIV-2 gp120 ectodomain the fibritin domain would stabilise the protein in a trimeric state.

For HIV-2_{ROD} similar constructs were generated and a further four truncated by 21 hydrophobic amino acids at the C-terminus of the gp36 ectodomain.

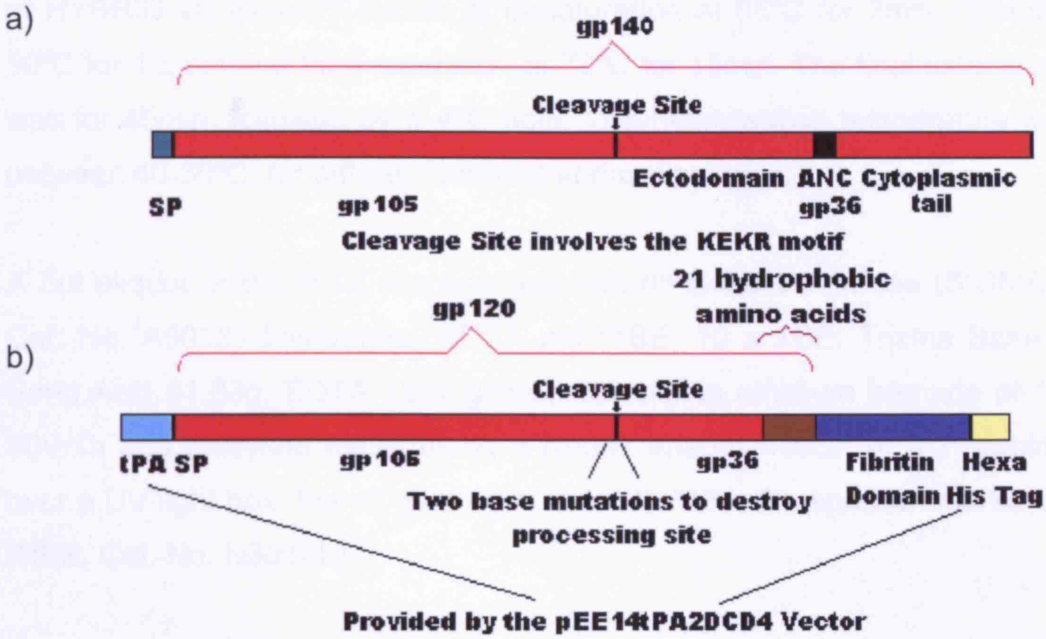


Figure 2.1: Glycoprotein Construct Schematic

a: Diagram of HIV-2 gp140 with the signal peptide (SP), SU (gp105) and the TM (gp36). Within the TM the ectodomain, membrane anchor (ANC) and cytoplasmic tail are indicated. b: Diagram of HIV-2 gp120 constructs with the longer tissue plasminogen activator SP (tPA SP), SU (gp105), TM (gp36) with the ANC and cytoplasmic tail removed to enable the production of soluble protein. The processing site mutations are indicated as well as the fibritin domain, His-tag and the 21 amino acid deletion in the gp120 'short' form.

2.1.4 PCR Generation of *env*-gene constructs

HIV-2 *env*-gene fragments (~2Kb) were recovered from existing pQ7 (Douglas et al., 1996) clones, derived from Caio patient samples, for cloning into pEE14tPA2DCD4. The fragments were amplified using PCR with native Pfu polymerase (Stratagene, Cat No. 600153). The 100µl PCR reaction mix contained 100ng of a particular clone, 0.1µM of both the forward and reverse primers (from 10mM stock) (Table 2.1) set up separately from the master stock of PCR reagents to prevent exonuclease activity of the Pfu polymerase. The master stock was prepared for x + 1 where x is the final number of reactions and it contained 1 x buffer (10 x stock supplied with Pfu polymerase from Stratagene), 0.25mM dNTP's (2.5mM stock, Pharmacia, Cat. No. 27-2035-01), 5 units per reaction of Pfu polymerase (2.5u/µl stock) made up to a final volume equivalent to 100µl per reaction with molecular biology grade autoclaved water. All PCRs were performed

on a MJ Research Cycler PTC-100 using the program HYBRID 20. The conditions of HYBRID 20 were 27 cycles of denaturation at 96°C for 2min, hybridisation at 50°C for 1.5min and then extension at 72°C for 15min. The final extension at 72°C was for 45min, followed by a 4°C hold. The hybridisation temperature was varied between 40-50°C, for different template/primer pairings.

A 5µl aliquot of the PCR reaction was run on a 0.8% Agarose (SIGMA, Type 1, Cat. No. A6013) Tris-borate, EDTA gel (TBE, 10 x TBE: Trizma Base 121.10g, Boric Acid 61.83g, EDTA 18.60g/litre), containing ethidium bromide at 1µg/ml, at 80V/1h and analysed either using a Kodak Imager 440CF or a Polaroid Camera over a UV light box. For all gels a Lambda DNA/*Bst* EII digestion marker was used (NEB, Cat. No. N3014L).

PCR products were purified by electrophoresis on a 0.5% Agarose (SIGMA, Type VII, Cat. No. A-4018) Tris-acetic, EDTA gel (TAE, 50xTAE: Tris Base 242.00g, Glacial Acetic Acid 57.10g, EDTA 18.612g/litre), containing ethidium bromide at 1µg/ml at 60V/90min in a 4°C cold room. Bands were excised and the DNA extracted using Ultrafree-MC Millipore filters (Millipore, Cat. No. UFC30HVNB) following manufacturers instructions, to yield 360µl aqueous phase. The latter was precipitated with 40µl 3M Sodium Acetate pH 5, 1µl glycogen (Sigma, Cat. No. G1765, stock concentration 10mg/ml) and 1ml of 96% ethanol.

Cleavage site mutants were generated by PCR splice overlap extension (SOE) using equivalent amounts of 5' (reaction 3) and 3' (reactions 4/5) templates with the required primer pairs (Table 2.1b) under the conditions described above. To 'stitch' the cleavage site mutants levels of template were adjusted to give equivalent amounts of 5' (3) and 3' (4/5) ends. All constructs were digested with *Bcl* I and *Eco*RI (Roche, Cat. No.'s 693979 + 1175084) to allow cloning into appropriately restricted pEE14tPA2DCD4.

a

Primer	F/R*	Sequence	Position[3]
H2NF	F	ATGATCTGATCATATGTGACTGTTTTCTATGGCRTACC	*Bcl* 1 – 32
H2REMR	R	GAACCCTAGCACRAACACACCTGTTGTATKTCTCACTKGAGCAGAGGAGTATCTTTCT TCTGRTGTAGGTGCGAAGCC	1419 – 1497
H2REMF	F	GGCTTCGCACCTACAYCAGAAGAAAGATACTCCTCTGCTCMAGTGAGAMATACAACAG GTGTGTTYGTGCTAGGGTTC	1419 – 1497
H2140	R	CACAAACGAATTCCTTGAATATACYTGAYCCAGGAGGTTAAGTCAAACCA	1999 – *Eco*R1
H2140FIB	R	CACAAACGAATTCCTAAAAAGGTAGAAAGTAATACCCATTCGCCATCTTTACGAACGT AAGCTTGCCCATCTCTTGGAGCTTCAGGAATATAACCTTGAATATACYTGAYCCAGGA GGTTAAGTCAAACCA	1999 – *Eco*R1
HINDF[1]	F	GACACGAAGCTTACAGGGCTG	*HIND* III -tPA signal
NEWR[2]	R	TGATGGTGAATTCCTTTTTGYAGYTCATACAT	1972- *Eco*R1
NEWFIB[2]	R	CACAAACGAATTCCTAAAAAGGTAGAAAGTAATACCCATTCGCCATCTTTACGAACGT AAGCTTGCCCATCTCTTGGAGCTTCAGGAATATAACCTTTTTGYAGYTCATACATATT CTT	1972 – *Eco*R1

[1]just before start of tPA signal

[2]truncate at YELQK to create constructs minus the 20 N-terminal amino acids

[3]Numbers based on HIV-2_{ROD} from Appendix Figure 2

b

Designation	1st round PCR	2nd round PCR	Designation
1	H2NF / H2140	N/A	
2	H2NF / H2140FIB	N/A	
3	H2NF / H2REMR	N/A	
4	H2REMF / H2140	H2NF / H2140	**N/34**
5	H2REMF / H2140FIB	H2NF / H2140FIB	**F/35**

c

Designation	Template	PCR
6	1	HINDF/NEWR
7	1	HINDF/NEWFIB
38	34	HINDF/NEWR
39	34	HINDF/NEWFIB

Table 2.1: PCR Primers

a: Primers used for PCR amplification of HIV-2 *env*-genes. * - The direction of each primer Forward (F) or Reverse (R) is shown. Sites recognized by *Bcl* I (TGATCA), *Eco*RI (GAATTC) and *Hind* III (AAGCTT) are underlined as are the positions in H2REMF/R that introduce mutations into the gp105/36 cleavage site. The position of each primer in the HIV-2_{ROD} *env*-gene is given. b: Primer pairings used in the generation of HIV-2 *env*-gene constructs. Second round PCRs, corresponding to PCR SOE to produce cleavage site mutants, paired product 3 with products 4/5 and the required primers as indicated. c: Templates and primer pairings used in the generation of the 'short' HIV-2 *env*-gene constructs. Templates were produced from the primer pairings in (b).

2.1.5 Plasmid

Plasmid pEE14tPAgp1203, containing the gene for HIV-1 IIIB gp120, was obtained from CellTech (now LONZA biologics) and supplied by P.E. Stephens (Jeffs et al., 1996) (Fig. 2.2). pEE14tPA encodes a human Cytomegalovirus (hCMV) promoter, a coding sequence for the tissue plasminogen activator (tPA) signal peptide and Glutamine Synthetase (GS) gene. The GS gene enables selection of positive cells during stable transfection in Chinese Hamster Ovary K1 (CHO K1) cells. The *env*-gene coding region was replaced with a fragment encoding a human 2 Domain CD4 construct carrying a His-tag at its C-terminus creating pEE14tPA2DCD4 (Daniels, unpublished). The latter plasmid was digested with *Bgl* II (Roche, Cat No. 348767) and *Eco*RI to allow cloning of the HIV-2 gp120 encoding *env*-genes.

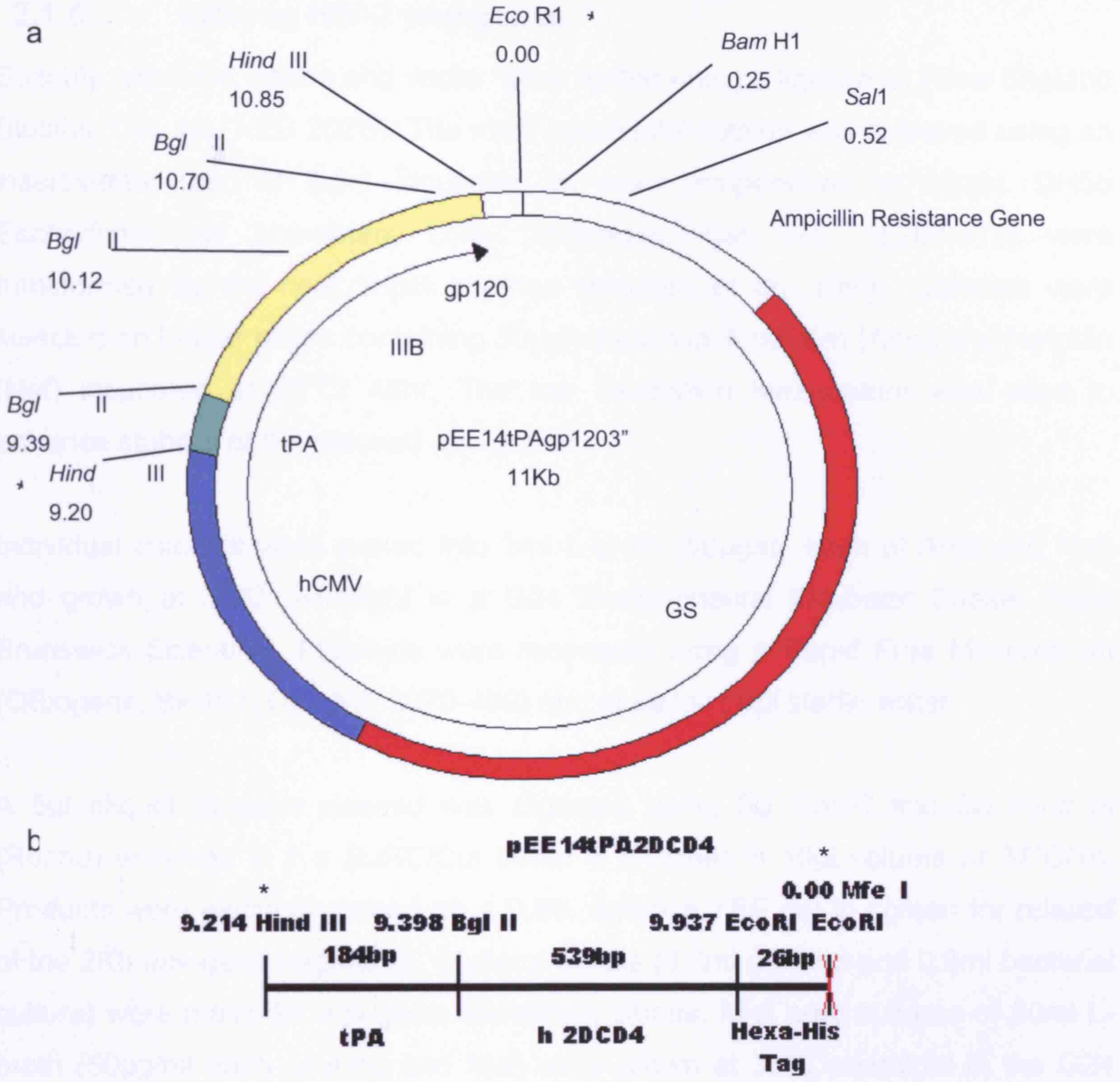


Figure 2.2: Plasmid Maps

a: Plasmid map of pEE14tPAgp1203 containing the HIV-1 IIIB gp120 gene. It also encodes for glutamine synthetase minigene (GS), human Cytomegalovirus (hCMV) promoter, tissue Plasminogen activator (tPA) signal peptide and contains an ampicillin resistance gene. b: Plasmid map of pEE14tPA2DCD4 encoding a human 2 domain CD4 gene and a hexa-His Tag. The *Eco*RI/*Mfe* I site at the origin was destroyed when this construct was produced and a new one created at the 5'-end of the hexa-His Tag coding region.* Indicates 'common' positions in the two pEE14tPA based plasmids.

2.1.6 Cloning HIV-2 *env*-genes

Suitably restricted inserts and vector were ligated using a ligation kit (New England Biolabs, Cat. No. NEB 202S). The most successful ligation was achieved using an insert/vector ratio of 2-8:1 incubated at room temperature for 30min. DH5α *Escherichia coli* competent cells (Invitrogen, Cat. No. 18263-012) were transformed by the heat shock method (Moulard et al., 1999). Colonies were selected on L-agar plates containing 50µg/ml each of Ampicillin (Amp) and Nafcillin (Naf) incubated at 30˚C/ 48hr. The low incubation temperature was used to enhance stability of the rescued *env*-genes.

Individual colonies were picked into 3ml L-broth (50µg/ml each of Amp and Naf) and grown at 30˚C overnight in a G24 Environmental Incubator Shaker (New Brunswick Scientific). Plasmids were recovered using a Rapid Pure Miniprep Kit (QBiogene, Bio101, Cat. No. 2070-400) and eluted in 50µl sterile water.

A 5µl aliquot of each plasmid was digested using 5U *Eco*RI and 5U *Hind* III (Roche) enzymes in 1 x SuRE/Cut buffer B (Roche) in 10µl volume at 37˚C/1h. Products were electrophoresed on a 0.8% agarose TBE gel to screen for release of the 2Kb *env*-gene fragments. Glycerol stocks (0.2ml glycerol and 0.8ml bacterial culture) were made for *env*-gene containing clones. Midi prep cultures of 50ml L-broth (50µg/ml each of Amp and Naf) were grown at 30˚C overnight in the G24 Environmental Incubator Shaker and processed with a Midiprep kit (QIAGEN, Cat. No. 12143). Plasmids were dissolved in 200µl sterile water and a 5µl aliquot was digested as before with 5U *Eco*RI and 5U *Hind* III and analysed on a TBE gel. The concentrations of plasmid stocks were quantified using the *Bio*photometer (Eppendorf, Cat. No. 6131 000.012) and then stored at -20˚C.

2.1.7 DNA Sequencing

HIV-2 *env*-genes in plasmids were sequenced using a gene walking approach with a series of primers (Table 2.2). Applied Biosystems Big Dye terminator v1.1 cycle sequencing kits (Cat. No. 4337450 and 4337036) were used. Reactions contained 200-400ng of plasmid DNA; 2pM primer; 2µl Big Dye and 2µl half reaction terminator and sterile water to a volume of 10µl. Thermocycling was carried out in a Peltier Thermal Cycler (PTC200 MJ Research) using 25 cycles of; denaturation 95˚C/30s; annealing 50˚C/15s; extension 60˚C/4min and a final hold at 4˚C. Sequencing products were purified by salt precipitation according to the ethanol/sodium acetate precipitation protocol, described in the MegaBACE sequencer protocols and air dried. Products were reconstituted in 10µl of MegaBACE loading buffer (Amersham Biosciences, Cat. No. 93-79916) and samples were run on an Amersham MegaBACE 1000 capillary sequencer. Resultant sequences were edited using pre Gap4 and Gap4 (Editing suite of computer programmes) (Flint et al., 1998), and aligned using the genetic data environment (GDE) suite of computer programmes (Smith et al., 1994).

Primer	Forward/ Reverse	Sequence	HIV-2 ROD*
P14F	Forward	ACCAGACATAATAGCTGACAG	pEE14tPA2DCD4
Y12	Forward	ATTCCCCTCTTTTGTGCAAC	46-65
Y8	Forward	GAGACATCAATAAAACCATG	211-230
Y11	Forward	TGTTACATGAACCATTGCAA	535-554
Y4	Forward	TGCACAAGGATGATGGAAAC	706-725
Y3	Forward	AATAGAACATATATATCTATTGG	767-789
Y1	Forward	ATGACTTGGTTCCTCAATTGG	1138-1158
Y6	Forward	TATTTGCCTCCTAGGGAAGG	1244-1263
Y9	Forward	ATGACATGGCAGGAATGGGA	1798-1817
Y10	Forward	ACCTGGAGGCAAATATCAGT	1832-1851
X1	Reverse	GGCAAGCACTGTATGGTTCCCCA	82-105
X17	Reverse	CATGGTTTTATTGATGTCTC	211-230
X2	Reverse	CATTGCTACACATAAGGG	244-261
X3	Reverse	CAAACCACATCTTTTGAGTACCA	481-503
X16	Reverse	TTGCAATGGTTCATGTAACA	535-554
X18	Reverse	ATAGCATCCCAATAGTGCTT	583-602
X4	Reverse	CCATGTGGAAGTTTGCGTTTCCAT	718-741
X13	Reverse	CCAATAGATATATGTTCTATT	769-789
X5	Reverse	CCAGCACCATGCTTGCCTGGG	940-960
X6	Reverse	CCAATTGAGGAACCAAGTCAT	1138-1158
X7	Reverse	GGTGCGAAGCCAATTGG	1408-1424
X8	Reverse	TGGAGGTTTTTCGTTCCCCA	1645-1664
X15	Reverse	GGTACAGTAGTGTGGCAGAC	1741-1760
X9	Reverse	CCATTCCTGCCACGTCAT	1798-1815
P14R	Reverse	GGTGTGGGAGGTTTTTTAAAG	pEE14tPA2DCD4

Table 2.2: Sequencing Primers

*The position of the primers in the *env*-gene of HIV-2_{ROD} are given (Appendix Figure 6). Primers P14F and P14R are located within pEE14tPA vector sequence to allow sequencing into the *env*-genes.

2.2 Screening for HIV-2 gp120 expression

2.2.1 Cells

Human Embryonic Kidney 293T cells (293T) (T stands for the SV40 T-Antigen introduced into the cell line) and African Green Monkey Kidney cells (CV-1) were passaged in Dulbecco's minimum essential medium (DMEM, Life Technologies 41966-029) supplemented with 10% heat inactivated foetal calf serum (FCS, PAA Laboratories Ltd, Cat. No. A15-245). Chinese Hamster Ovary K1 (CHO K1) cells, used to produce stable cell lines, were maintained in CHO GS medium (CHO K1 medium with glutamine supplements – without glutamine) supplemented with 10% heat inactivated dialysed FCS (HD FCS) and Penicillin/Streptomycin (P/S) (100units/100µg /ml, Sigma, Cat. No. P0781) Glutamine (2mM – made in house) or L-Methionine Sulphoximine (MSX, 200µM, Sigma, Cat. No. M5379-1G). CHO K1 cell lines that express HIV-2 gp120 constitutively, were maintained in CHO IIIA medium (Invitrogen, Formulae No. 97-0147DK) supplemented with 1% HD FCS, 2% HT Supplement (Invitrogen, Cat. No. 41065-012), P/S (100units/100µg/ml) and 200µM MSX (concentration determined by previous kill curves). All cell lines were incubated at 37°C in a 5% CO_2 atmosphere incubator. Cell lines were cryopreserved in 50% FCS, 40% DMEM/RPMI and 10% DMSO, initially at -70°C followed by storage in Liquid Nitrogen.

2.2.2 Transient Expression

pEE14tPA plasmids carrying HIV-2 *env*-genes were assayed for expression competence in 293T cells, using one of the following transfection methods, which are briefly explained below.

2.2.2.1 Calcium Phosphate Transfection

The day prior to transfection, the appropriate number of 60mm dishes were seeded with 2 x 10^6 cells each. The dishes were then incubated overnight at 37°C.

On the day of transfection DMEM with 10% FCS was prewarmed in a 37°C waterbath. The medium covering the cells in the 60mm dishes was removed and 4ml of fresh medium was added to each dish. The cells were then incubated at 37°C for 3h.

6µg of DNA (xµl), water (H_2O) (263µl – xµl) and Calcium Chloride ($CaCl_2$) (37µl) were mixed together in an eppendorf to give a final volume of 300µl. This DNA mix was then added to a bijou containing 2 x HEPES-buffered saline (HBS) (300µl) to give a total volume of 600µl; the HBS was vortexed gently as the DNA mix was added. Following mixing the DNA/HBS mix was left for 30min at room temperature. Before the DNA/HBS mix was added drop by drop onto the dishes it was briefly vortexed. To ensure even distribution the dishes were rocked from side to side. The dishes were incubated at 37°C for 72hr.

2.2.2.2 Lipofectamine Transfection

The day before transfection, the appropriate number of 60mm dishes were seeded with 2 x 10^5 cells each. The dishes were then incubated overnight at 37°C.

On the day of transfection the following was prepared in 12mm x 75mm sterile tubes. 1µg of DNA was diluted into 100µl serum free medium (OPTI-MEM I reduced serum medium gives optimal results – GIBCO, Cat. No. 31985-047) per transfection and 5µl (the protocol states that Lipofectamine can be used in the range of 2-25µl, however we found 5µl to be adequate) of Lipofectamine reagent was diluted into 100µl serum free medium per transfection.

The two solutions were combined, mixed gently and incubated at room temperature for 30min, this allowed DNA liposome complexes to form. While the complexes formed, each dish of cells was washed once with 4ml of serum free medium.

0.8ml of serum free medium was added to each transfection tube containing the complexes. The diluted complex solution was then mixed gently and overlaid onto the washed cells. The cells were then incubated with the complexes for 2h at 37°C in a CO_2 incubator.

Following incubation 1ml of growth medium was added containing twice the normal concentration of serum. The medium was replaced with fresh complete medium at 24h following the start of transfection and then the incubation was continued for a further 48h.

2.2.2.3 Effectene Transfection

The day prior to the transfection, 60mm dishes were seeded with 2 x 10^5 cells each and then the dishes were incubated overnight at 37°C.

On the day of transfection, 1µg of DNA, dissolved in sterile autoclaved water, was diluted with the DNA condensation buffer, Buffer EC, to a total volume of 150µl. 6.4µl of enhancer was added to the mix which was then vortexed for 1s. Following an incubation of the mixes at room temperature for 5min they were centrifuged (10s 10,000rpm) briefly to collect the mixture at the bottom of the tube.

20µl of effectene transfection reagent was added to the DNA-enhancer solution, this was then mixed by pipetting up and down five times. The samples were incubated for 5-10min at room temperature, which allowed complex formation. While complex formation took place, the growth medium was gently aspirated from the dish and the cells were washed once with PBS. 4ml of fresh growth medium (can contain serum and antibiotics) was added to the cells.

1ml of cell growth medium (can contain serum and antibiotics) was added to the reaction tube containing the transfection complexes, this was then mixed by pipetting up and down twice. The transfection complexes were then added immediately, drop wise, onto the cells in the 60mm dishes. The dish was then gently rocked to ensure uniform distribution of the complexes.

The cells were incubated with the complexes at 37°C and 5% CO_2 for 72h.

2.2.2.4 Superfect Transfection

The day prior to the transfection, 60mm dishes were seeded with 2 x 10^5 cells/dish and then incubated overnight at 37°C.

1.25µg of DNA were added to 150µl of growth medium (without serum and antibiotics), this was then briefly vortexed and centrifuged (10s, 10,000rpm). 6.25µl of Superfect reagent was added to the DNA solution, which was then mixed by repeated pipetting (see above). The samples were incubated at room temperature for 5-10min. While transfection complexes formed, the target cells were washed once with PBS.

1ml of growth medium (with serum and antibiotics) was added to the reaction tube, which was then mixed and transferred to the cells. Following an incubation of 3h, the medium was removed and the cells were washed three times in PBS. Fresh growth medium was added and the dishes were incubated for 72h at 37°C in a 5% CO_2 incubator.

2.2.2.5 Cell Lysis and Harvesting

At 72h the tissue culture supernatant (TCSN) was removed from the transfected cells and stored in 2ml Sarstedt tubes at -20°C for later screening for secreted HIV-2 gp120. The cells were incubated at room temperature for 5-10min in 200µl RIPA lysis buffer (150mM sodium chloride, 50mM Tris HCl pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate (Sigma, Cat. No. D-6750), 0.1% SDS (BioRad, Cat. No. 161-0418)). The lysates were transferred to Sarstedt tubes and until screened for HIV-2 gp120 expression stored at -20°C.

2.2.3 Stable Expression

Attempts were made to establish CHO K1 cells which constitutively express HIV-2 envelope proteins (based on (Davis et al., 1990)).

2.2.3.1 Transfection

One day before transfection cells were transferred to glutamine free medium. The transfection procedure used was the same as that described above for the effectene method. After 72h TCSN was removed and stored at -20°C prior to screening for secreted HIV-2 gp120, and cells were rinsed with 400µl trypsin versene (8.00g NaCl, 0.20g KCl, 1.15g Na_2HPO_4, 0.20g KH_2PO_4, 0.10g EDTA, 1.25g Trypsin, 0.01g Phenol Red /litre adjust pH to 7.8.) to remove any remaining medium. Cells were then trypsinised (200µl for 5-10min at 37°C) and 800µl of CHO GS medium added to inactivate the trypsin and resuspend the cells. Two thirds of the cells were cryopreserved and the remaining cells passaged into one well of a six well plate. The cells were fed with fresh medium containing 200µM MSX at this time and re-fed every 2-3 days with fresh MSX-containing medium. Cultures were monitored for cell death and the formation of cell colonies.

2.2.3.2 Selection of Cloned Transfectants

When colonies appeared two methods were employed to isolate individual colonies.

2.2.3.2.1 Individual Clone Selection

The colonies were marked on the dish and then picked manually using precut and autoclaved filter paper and forceps. Each filter was then transferred into a well of a 24 well plate containing 2ml of fresh MSX-containing medium.

2.2.3.2.2 Dilution Method

The dilution method involved trypsinising the wells containing the cell colonies, cell counting using a haemocytometer and dilution of the cells to 100, 10 or 1 cell per well in 200µl of fresh MSX containing medium in a 96 well plate.

For both methods potentially positive cells were maintained and expanded to give confluency in a 60mm dish, at which time TCSNs were stored for western blot screening and the cells cryopreserved.

2.2.4 Antibodies

A range of monoclonal antibodies and polyclonal antisera raised against HIV-2 glycoprotein were collected to allow screening for recombinant gp120 products by a variety of methods (described below). Polyclonal serum raised against HIV-2_{ST} gp105 (NIH 1410) (Ivey-Hoyle et al., 1991; Mulligan et al., 1992a) was used for detection of HIV-2 gp120/gp105 at a dilution of 1:5000 in western blotting. A goat anti-rabbit peroxidase conjugate (SIGMA, Cat. No. A-6154) was diluted 1:5000 to detect NIH 1410. Two monoclonal antibodies used to detect HIV-2 gp120/gp105 were raised against HIV-2_{ROD} (ARP 3032 and ARP 3030, CFAR) (Sattentau et al., 1993). The secondary antibody to ARP 3032 and ARP 3030 was an anti-mouse IgG Horse Radish peroxidase conjugate (Promega, Cat. No. W402B 1mg/ml) used at a dilution of 1:5000 for western blotting. Nine further monoclonal antibodies used in the detection of HIV-2 gp120/gp105 were raised against recombinant HIV-2_{ROD} gp105 produced by recombinant baculovirus in insect cells (ARP 3083 – ARP 3091, CFAR) (McKnight et al., 1996). The secondary antibody to ARP 3083 – ARP 3091 was an anti-rat IgG (whole molecule) peroxidase conjugate (SIGMA, Cat. No. A 9037) used at a dilution of 1:1000 for western blotting. In an attempt to detect HIV-2 gp36 three monoclonal antibodies were obtained two (ARP 3002 and ARP 3044, CFAR) were raised against a conserved region within SIV (Kent et al., 1991; Kent et al., 1992) and one was raised against the peptide AIEKYLKDQAQLNAWGCAFRQVC (Abcam, Cat. No. ab13412) representative of one of the principal immunogenic regions within the N terminal region of gp36. The secondary antibody to these three anti-gp36 antibodies was an anti-mouse IgG

Horse Radish peroxidase conjugate (Promega, Cat. No. W402B 1mg/ml) used at a dilution of 1:5000 for western blotting (Table 2.3).

Primary Antibody	Clonality	Host	Raised Against	Dilution	Secondary Antibody	Dilution
NIH 1410	Polyclonal	Rabbit	HIV-2 ST gp105	1/5000	Goat anti Rabbit HRP	1/5000
Preabsorbed NIH 1410 *	Polyclonal	Rabbit	HIV-2 ST gp105	1/1000	Goat anti Rabbit HRP	1/5000
ARP 3030	Monoclonal	Mouse	HIV-2_{ROD} gp105	1/2500[1]	Anti mouse HRP	1/5000
ARP 3032	Monoclonal	Mouse	HIV-2_{ROD} gp105	1/2500	Anti mouse HRP	1/5000
ARP 3083	Monoclonal	Rat	HIV-2_{ROD} gp105	Various	Anti rat HRP	1/1000
ARP 3084	Monoclonal	Rat	HIV-2_{ROD} gp105	Various	Anti rat HRP	1/1000
ARP 3085	Monoclonal	Rat	HIV-2_{ROD} gp105	Various	Anti rat HRP	1/1000
ARP 3086	Monoclonal	Rat	HIV-2_{ROD} gp105	Various	Anti rat HRP	1/1000
ARP 3087	Monoclonal	Rat	HIV-2_{ROD} gp105	Various	Anti rat HRP	1/1000
ARP 3088	Monoclonal	Rat	HIV-2_{ROD} gp105	Various	Anti rat HRP	1/1000
ARP 3089	Monoclonal	Rat	HIV-2_{ROD} gp105	Various	Anti rat HRP	1/1000
ARP 3090	Monoclonal	Rat	HIV-2_{ROD} gp105	Various	Anti rat HRP	1/1000
ARP 3091	Monoclonal	Rat	HIV-2_{ROD} gp105	Various	Anti rat HRP	1/1000
ARP 3002	Monoclonal	Mouse	SIV gp41	1/2000	Anti mouse HRP	1/5000
ARP 3044	Monoclonal	Mouse	SIV gp41	1/2000	Anti mouse HRP	1/5000
HIV-2 gp39 Abcam	Monoclonal	Mouse	HIV-2_{ROD} gp39	1/1000	Anti mouse HRP	1/5000

Table 2.3: Antibodies used in Western Blotting

The antibody pairings and dilutions used for western blot procedures are shown. *See Appendix – Method 1 for Preadsorbed Antibody method by aldehyde activated porus silica.[1] when using 'in house' prepared hybridoma TCSN a 1/10 dilution was used.

2.2.5 Polyacrylamide Gel Electrophoresis (PAGE)

Lysates were clarified by centrifugation at 15,700 x g/5min (MicroCentaur) prior to gel preparation. 15µl of either cleared cell lysate or TCSN in 1 x sample loading buffer (50mM Tris pH 6.8, 5% mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) were denatured at 100°C/5min in a Grant BT3 heater and then loaded on to SDS 5-15% gradient polyacrylamide gels (Appendix – Method 3). Samples were electrophoresed at 200V/45min in 1 x SDS PAGE running buffer (50mM Tris, 384mM Glycine and 0.1% SDS/litre). Molecular weight markers used were either See Blue (Invitrogen, Cat. No. LC5925), or Prestained SDS-PAGE Standards, Broad Range (BIO-RAD Cat. No. 161-0318).

2.2.6 Western Blotting

The positive control used in western blots was EVA 621 (CFAR) recombinant HIV-2_{ROD} gp105 produced by recombinant baculovirus or TCSN from CHO K1 cells constitutively expressing construct 39 (designated 39.5 8) generated in the course of work for this thesis. Negative controls, cell lysates and TCSN, for transfection assays were vector-transfected only and non-transfected cells. Proteins on polyacrylamide gels were transferred to Hybond C nitrocellulose membrane (Amersham Biosciences, Cat. No. RPN303E) using a Trans-Blot SD semi-dry transfer cell (BioRad) at 15V/70min. Membranes were blocked using 10% milk powder (Marvel, Western Lab Supplies, Cat. No. U599) in PBS/0.1% v/v Tween20 (PBT) for 30min at room temperature. Membranes were then probed with the relevant antibody (Table 2.3), in 10% Marvel in PBT incubating at room temperature for 60min or overnight at 4°C. Membranes were washed three times with PBT for 10min each and then probed with an appropriate secondary antibody (Table 2.3), in 10% Marvel in PBT at room temperature for 30min. Membranes were washed three times with PBT for 10min each and then treated with enhanced-chemiluminescence (ECL) development reagent (2ml of each reagent for 15s, Amersham Pharmacia Biotech, Cat. No. RPN2106). Membranes were then exposed to XBM film (X-ray Retina, Cat. No. 100NIF) and machine (FPM 3800A, FujiFilm) processed in a dark room.

2.2.6.1 Dot Blot Method

Potential gp120 containing samples were dotted directly onto nitrocellulose membrane in two 5µl drops, and allowed to dry. Membranes were then blocked with 10% Marvel in PBT and probed as described for the PAGE-based western blot above.

2.2.7 Immunoprecipitation (IP)

Immunoprecipitation was used to aid in the determination of the choice of antibody for immunoaffinity chromatography. One millilitre of TCSN was precleaned (removal of cross reacting contaminants from TCSN) by adding 50µl of rabbit serum (SIGMA, Cat. No. R9133), which was then incubated on ice for 1h. During the incubation 100µl of SAC (Protein A Staphlycoccus aureus SIGMA, Cat. No. P-7155) was taken and washed three times with 500µl of PBS (following each wash the sample was centrifuged briefly (10,000 x g 30s) and the supernatant removed) finally the SAC was resuspended in 100µl of PBS and stored on ice.

The washed SAC was added to the TCSN/rabbit serum mix, incubated on ice for 30min and then centrifuged at 10,000 x g/15min (Eppendorf Centrifuge 5415R, Eppendorf GMBH, Germany) at 4˚C. The supernatant was removed and 50µl was added to each tube containing 5µg of antibody followed by 1h incubation on ice, enabling immune complex formation. 50µl of a washed (as above for SAC) 10% protein G slurry was added to each tube, followed by incubation at 4˚C for 1h on a suspension shaker (Model 802, Luckham).

The samples were then centrifuged at 10,000 x g/5min. The supernatant was discarded and the pellet was washed twice with PBS. The pellet was frozen for later analysis by SDS PAGE followed by western blot.

2.2.8 Immunofluorescence

To assess what proportion of CHO K1 cells in each cloned cell-line were expressing gp120, immunofluorescence was used. At least 1 day before an experiment, cells were plated at 1-2 x 10^5/ml on to glass coverslips in either 24 well or 12 well plates.

On the day of the experiment, microscopic inspection was used to confirm that the cells that had adhered to the glass slides were 80% confluent. Cells were washed twice with PBS, 1ml/well, by gently running the buffer onto the side of the well and then fixed by adding 1ml of 4% Paraformaldehyde/PBS and incubating for a minimum of 20min at room temperature.

Wells were washed three times with PBS as before, ensuring that the coverslips were not allowed to dry. For permeabilisation of cell membranes, 1ml of 0.2% TritonX100/PBS per well was used and incubated for 30min. Cells were then washed with PBS as above and non-specific binding sites were blocked with 3% BSA/PBS for 30min at room temperature.

Primary antibody solutions, using purified antibody, were prepared as a 1:50-1:100 dilution in 1% BSA/PBS (dilution buffer), depending on the concentration of the antibody used, 200µl of each primary antibody solution was used to cover the cells. The cells were washed three times with PBS and then incubated with the primary antibody for 1h at room temperature on an orbital shaker (S05 - Stuart Scientific). The cells were washed again with PBS as above and incubated on the shaker for 30min with a labelled (FITC or other fluorescent dye) secondary, species-specific antibody at a 1:200 dilution. During this incubation period the plate was covered with aluminium foil. DAPI (1µg/ml) was prepared at the end of the incubation, in order to stain cell nuclei. The DAPI was added to the solution in the wells and incubated for a further 1min, then the cells were washed with PBS as before.

Coverslips were washed thoroughly by repeated immersion into a beaker of water, then dried by touching a piece of tissue/filter paper to one edge. The cleaned coverslips were mounted on the slides by placing a drop (i.e. 5µl) of citifluor (Citifluor Ltd, Cat. No. AF1) onto a slide and then gently lowering the coverslip, cell-side down, onto the drop of citifluor The excess citifluor was removed with a paper towel, by gently pressing down the cover slip. The slides are then stored in the dark at 4°C.

2.3 HIV-2_{ROD} gp120 Capture Systems

Various chromatographic procedures were investigated, including the use of affinity, ion exchange and gel filtration media, which required use of a P1 pump system to ensure controlled flow rates. Before and at the end of a column being attached to a P1 pump the tubing was rinsed by flowing approximately 10ml of 70% ethanol followed by 10ml of ultrapure water through the pump.

2.3.1 Concentrating Protein

TCSN in volumes ranging from 20 litres (for Hybridoma TCSN) to 2ml (fractions from the FPLC column) required concentration to workable volumes, the methods differed depending on the volume of the original TCSN.

2.3.1.1 Tangential Flow Filtration

For large volumes, more than 6 litres, the TCSN was clarified by centrifugation (3000 x g, 10min, Beckman J6-HC), and then pumped through a 0.45 micron cross flow membrane filter (Sartorius Corporation, Cat. No. 3051860601W). The clarified supernatant was then concentrated using a 30KDa molecular weight cut-off (MWC) membrane (Sartorius Corporation, Cat. No. 3051445901E). The clarification and concentration was carried out using a Sartocon® Slice System (Sartorius Corporation, Cat. No. 17521---001). The resultant ~400ml of concentrate had 0.02% sodium azide added to it before storage at 4°C until use.

Small volumes of clarified supernatant (3-6 litres) were concentrated using a 10KDa MWC hollow fiber filter (Amersham Biosciences, Cat. No. UFP-10-C-4X2MA). The concentration was carried out using a QuixStand benchtop system (Amersham Biosciences, Cat. No. QSM-02S) and the concentrate, ~ 100ml, had 0.02% sodium azide added to it before storage at 4°C until use.

2.3.1.2 Stirred Cell

Used for small volumes, of less than 1 litre. The appropriate sized filter (usually 10/30KDa) was placed in the stirred cell (ChemLab, C400), leaks in the device were checked for by using distilled water.

A funnel was used to add the TCSN into the stirred cell and then the lid was tightened. The stirred cell was connected to stage 1 (Nitrogen Cylinder), the valve opened and the pressure set to 2 bar. The flow through was collected in a beaker.

When the TCSN had reduced to a workable volume (~50ml) the valve was closed and the pressure released. Stage 1 was unscrewed and the concentrated TCSN removed from the stirred cell.

2.3.1.3 Filter centrifugation

For volumes of 20–100ml a Vivacell 70 was used, which has a 30KDa filter (Vivascience, Cat. No. VS6022). The sample to be concentrated was placed in the filter unit and then the pressure head (VCA700) was attached to the concentrator assembly and tightened by hand. The APC regulator was set to 5 bar, the valve opened and the assembly attached to the female coupling to charge the pressure head. The coupling was detached by raising the outer locking sheath. The sample was left to concentrate at room temperature.

For volumes of 2–20ml a Vivaspin 6ml concentrator with a 10KDa filter (Vivascience, VS0601), was used. The sample to be concentrated was placed in the filter and then the assembly was centrifuged at 2,000 x g (IEC Centra-4R, Damon, UK).

For volumes of 0.25–2ml a Vivaspin 500, with either 10KDa (Vivascience, VS0102) or 30KDa (Vivascience, VS0121) filters, was used depending on the MW of the protein which was to be concentrated. The sample was placed in the filter and concentrated using an Eppendorf Centrifuge 5415R (Eppendorf GMBH, Germany) at 10,000 x g. Periods of centrifugation were adjusted to give the desired concentration effects.

2.3.2 Bradford Reagent

Bradford reagent (5 x stock 50mg Coomassie Brilliant Blue G-250, 25ml ethanol, 50ml 85% (v/v) Orthophosphoric acid, 25ml ddH_2O) was used as a crude method for determining the presence or absence of protein. 10µl of sample was added to 90µl of Bradford Reagent, if protein was present the Bradford Reagent would turn from a brown colour to a blue colour.

2.3.3 Desalting Column

PD-10 Desalting Columns (Amersham Biosciences, Cat. No. 17-0851-01) were used to buffer exchange small samples quickly. Manufacturer's protocols were followed.

Briefly excess fluid was allowed to drain through the column. The column was then equilibrated using 25ml of the appropriate elution buffer. The flow through was discarded. The sample to be buffer exchanged was added to the column in a total volume of 2.5ml. The flow through was discarded. The protein was eluted using 3.5ml of the required buffer, the flow through was collected and stored at 4°C until required.

2.3.4 HIS Columns

All constructs made contained a C-terminal hexa-His tag which I hoped to use for gp120 purification.

2.3.4.1 Nickel

Two millilitres of Ni-NTA HIS-bind resin were used to give a 1ml column. The Ni-NTA HIS-bind resin (Novagen, Cat. No. 69670-3) was washed with 10 x column volume of binding buffer (0.5M NaCl, 20mM Tris HCl, 5mM Imidazole pH 7.9).

5ml of concentrated TCSN was passed through a 0.45µm filter and its pH adjusted to 7.9, before addition to the column. The flow through was collected at this stage and all the subsequent steps. The column was then washed with 10 x column volumes of binding buffer. It was then washed with 6 x column volumes of wash buffer (0.5M NaCl, 60mM Imidazole, 20mM Tris HCl pH 7.9). To elute the protein 6

x column volumes of elution buffer (1M Imidazole, 0.5M NaCl, 20mM Tris HCl pH 7.9) were used and the flow through was collected in 1ml fractions.

The column was stripped of its charge using 6 x column volumes of strip buffer (0.5M NaCl, 100mM EDTA, 20mM Tris HCl pH 7.9) and then recharged with 6 x column volumes of charge buffer (50mM $NiSO_4$). Finally, the Nickel column was stored in 20% ethanol at 4°C.

To address the issue of His-tag exposure TCSN was denatured using 8M urea, to see if the hexa-His tag was more available for binding. It has been suggested that under denaturing conditions the hexa-His tag should be fully exposed, thereby improving binding to the Nickel and reducing non-specific binding (The QIAexpressionist™ – Fifth Edition, Qiagen). The nickel column was run in the same way as above, except for the addition of 8M urea at each step.

2.3.4.2 Cobalt

The methodology employed was the same as for the Nickel column, but with different buffers. The concentrated TCSN was adjusted to pH 8 and filtered through a 0.45µm filter. Binding buffer was adjusted to pH 8 containing 50mM NaH_2PO_4, 10mM Tris HCl, 100mM NaCl; wash buffer was adjusted to pH 7 containing 50mM NaH_2PO_4, 100mM NaCl; and elution buffer at pH 5-5.3 contained 50mM NaH_2PO_4, 20mM PIPES and 100mM NaCl.

2.3.5 Ion Exchange Chromatography (IEX)

Ion exchange chromatography was used to capture HIV-2 gp120 on the basis of charge. The first step in IEX was to decide whether an anion, Q Sepharose fast flow (1ml), or a cation, SP Sepharose fast flow (1ml) (Amersham Biosciences, HiTrap™ IEX Selection Kit, Cat. No. 17-6002-33), exchanger was required. To do this, columns were run with a range of different pHs to determine which conditions to further optimise. Based on the amino-acid sequence of HIV glycoprotein ectodomain their isoelectric point (pI) were predicted to fall in the range pH 7-8 (ProtParam tool http://nimri.nimr.mrc.ac.uk/webselect/ifmolbio.htm and Sednterp - see section 2.4.9), but others have measured pI in the acid range (~pH 5)

presumably due to the high sialic acid content of the glycoprotein carbohydrate component (Zhang et al., 2002). Therefore I investigated the pH range of 4-9.

Columns were washed with 5ml of start buffer (See section 2.6.14.9), followed by 5ml of elution buffer.

To equilibrate a column, 5ml of start buffer was used. 4ml of TCSN was then applied (at the appropriate pH), and the column was washed using 5ml of start buffer, with the flow through being collected at each stage.

To elute bound protein 5ml or more of elution buffer was used, until the Bradford result was negative. To collect any HIV-2 gp120 that was still bound to the column 5ml of 2M sodium chloride was used and the flow through collected. The column was washed with 5ml of water and stored in 20% ethanol at room temperature.

2.3.6 Lectin Affinity Chromatography

Due to the highly glycosylated nature of the HIV glycoprotein, lectin affinity chromatography has been used extensively by others in Env purification protocols (Gilljam, 1993; Jeffs et al., 2004; Sourial et al., 2005). A range of lectins of known specificity (Table 2.4) were used in small columns (1ml) first to assess which would be most useful for our HIV-2 gp120.

Lectin	Concanavalin A	*Galanthus Nivalis* Lectin	*Hippeastrum* Hybrid Lectin	*Pisum Sativum* Agglutinin
Abbreviation	Con A	GNA	HHL	PSA
Isolated From	*Canavalia ensiformis* (Jack Bean) seeds	Snowdrop Bulbs	Amaryllis bulbs	*Pisum sativum* (garden pea) seeds
Specificity	α-linked mannose	(α-1,3) mannose	(α-1,3) and (α-1,6) mannose	α-linked mannose, with an N-acetylchitobiose-linked α-fucose residue in the receptor sequence
Provided as	5ml Sepharose Bound preserved in 20% ethanol	5ml Agarose Bound	5mg uncongugated	2ml Agarose bound
Company	Amersham Biosciences	Vector Laboratories	Vector Laboratories	Vector Laboratories
Cat. No.	17-0440-03	AL-1243	L-1380	AL-1053

Table 2.4: Lectin Information Guide

2.3.6.1 Concanavalin A

The column was rinsed with 20% ethanol and then equilibrated with 5 column volumes (5ml) of binding buffer (20mM Tris HCl, 0.5M NaCl at pH 7.4). 2ml of concentrated TCSN was clarified by centrifugation (5min 10,000g) and filtration using a 0.45μm filter, and the pH adjusted to 7 before it was run over the column. The flow through was collected. This was followed with 5 column volumes of binding buffer and 5 column volumes of elution buffer (20mM Tris HCl, 0.5M NaCl, 1M Methyl α D Glycopyranoside at pH 7.4). All the flow throughs were collected with the elution buffer flow through being collected in 1ml fractions.

The resin was suspended in 10ml of elution buffer and placed at 50°C for 1h. Following incubation, the elution buffer was collected as one fraction. Elution buffer was used to rinse sides of column for extra resin. This was collected in fractions until the Bradford reagent no longer turns blue

The resin was stored in storage buffer (1M NaCl, 1mM $CaCl_2$, 1mM $MnCl_2$, 1mM $MgCl_2$, 0.1M Acetate Buffer at pH 6). All fractions were stored at 4°C or -20°C depending on the length of time between running the column and assaying.

2.3.6.2 *Galanthus nivalis*

The column was equilibrated with 5 column volumes of binding buffer (PBS). The TCSN was centrifuged and filtered as above and run over the column three times. The column was washed in wash buffer (PBS with 0.04M α-methyl mannoside) until the Bradford reagent no longer turned blue. Bound protein was eluted with 0.8M α-methyl mannoside in PBS in 1ml fractions for 5 column volumes or until Bradford reagent no longer turned blue. The resin was stored in PBS containing 0.02% sodium azide. The fractions were stored at 4°C or -20°C depending on the time delay until assaying.

2.3.6.3 *Pisum sativum* agglutinin

Method as for GNA (above)

2.3.6.4 *Hippeastrum* hybrid

Method as for GNA (above)

2.3.7 Monoclonal Antibody based Immunoaffinity Chromatography

Monoclonal antibody based immunoaffinity chromatography had the possibility of providing single step purification due to the nature of the antibody's specifity. In addition, it was considered that the use of an antibody that was conformation dependant might allow the purification of correctly folded HIV-2 gp120.

2.3.7.1 Hybridomas

In total, eleven hybridoma cell lines were obtained from CFAR with kind permission of their generators Dr Q. Sattentau and Dr A. McKnight. The CFAR catalogue numbers and information on the epitopes recognised by the antibodies produced are provided (shown in Table 2.5).

CFAR Designation	Epitope Domain	Epitope	Source
ARP 3030	C1	unknown	(Sattentau et al., 1993)
ARP 3032	C2	unknown	(Sattentau et al., 1993)
ARP 3083	C1	DDYQEITLNVTE	(McKnight et al., 1996)
ARP 3084	V1	SEDTPCARA	(McKnight et al., 1996)
ARP 3085	V2	GEEETINCQ	(McKnight et al., 1996)
ARP 3086	V2	FNMTGL	(McKnight et al., 1996)
ARP 3087	Not V1/2/3 c. d.	unknown	(McKnight et al., 1996)
ARP 3088	Not V1/2/3 c. d.	unknown	(McKnight et al., 1996)
ARP 3089	Not V1/2/3 c. d.	unknown	(McKnight et al., 1996)
ARP 3090	V3	LMSGHVFHSHYQ	(McKnight et al., 1996)
ARP 3091	V3	SGHVFHSHYQ	(McKnight et al., 1996)

Table 2.5: Hybridoma cell lines obtained from CFAR

Catalogue numbers relating to the antibodies produced by CFAR (NIBSC, UK) are given together with information about the epitopes that the antibodies recognise on gp105 and who produced the hybridomas originally. c.d. conformational dependant epitopes, C – constant domain, V – Hypervariable domain.

2.3.7.2 Maintenance of Hybridomas

2.3.7.2.1 Small Scale Maintenance

The majority of hybridoma cell lines were maintained in DMEM (GIBCO, Cat. No. 41965-039) containing 10% FCS and 1:100 P/S. However, ARP 3083, 3084, 3089 and 3090 did not thrive in DMEM and so were grown in RPMI 1640 and L-Glutamine used at a concentration of 2mM (GIBCO, Cat. No. 21875-034) with 10% FCS and 1:100 P/S. To encourage the hybridomas to replicate interleukin 6 (IL-6) (Roche, Cat. No. 1 444 581) was added at a concentration of 1ng/ml (100u/ml). Hybridoma Cell lines were cryopreserved as stated in Section 2.2.1.

2.3.7.2.2 Large Scale Production of Hybridoma Supernatant

Cell lines ARP 3030, ARP 3032 and ARP 3085 were all grown in DMEM medium, with 10% FCS, and the following scale-up procedure was performed by members of the large scale laboratory, NIMR.

For each hybridoma, 4 medium flasks ($75cm^2$) were grown to confluency in a 37°C with 5% CO_2.

The contents of a set of medium flasks were transferred into a 1 litre spinner bottle (Bellco, Cat. No. 1965-01400), and medium including FCS was added up to the 250ml mark. The 1l bottle was placed back in the CO_2 incubator with port caps on spinners loosened and the cultures stirred at 25rpm.

The cells were allowed to replicate for 3-5 days (confluency) at which time the volume was increased to 500ml. Following another 3-5 days of incubation the volume was increased to 1 litre.

At a concentration of 1 x 10^6 cells/ml the 1 litre of cells in the spinner bottle was split into two 1 litre spinner bottles.

Following a further 3-5 days, 1 litre of cells was added to large spinner vessels (5-8 litre), and these were made up to 3 litres with medium containing 5% FCS. These large vessels were placed in a 37°C incubator which was not gassed with CO_2.

Following another 3-5 days, the cultures were made up to a final volume of five litres using medium containing 5% FCS, to yield a final concentration of FCS of approximately 2-3%. The cells were allowed to replicate for a further 7 days. The TCSN was harvested and concentrated using the Sartocon® Slice System described above, to approximately 400ml.

2.3.7.3 Purification of Antibodies

The concentrated TCSN was placed in a beaker with a magnetic stirring bar. The appropriate amount of ammonium sulphate (27g/100ml) was weighed out. The beaker was placed on a stirrer in a 4°C room, the ammonium sulphate was added gradually over 30min with continuous slow stirring. When all the ammonium sulphate had dissolved, the TCSN was left gently stirring for another 30min. The mixture became opaque indicating that protein had precipitated.

The TCSN was then aliquoted into 250ml conical centrifuge tubes and precipitates were collected by centrifugation at 3,400 x g/30min at 4°C in a Beckman J6-HC Centrifuge (Beckman) on a brake 5 setting.

Plastic rods were used to break up the pellets, which were resuspended in approximately 15ml of 1 x PBS by repeated pipetting. The resuspended pellets were dialysed at 4°C overnight against 4.5 litres of 1 x PBS. The PBS was then changed and after 4-5h the TCSN was removed from dialysis tubing and stored in appropriate sized containers ready for protein G column purification.

A 5ml HiTrap Protein G HP column (Amersham Biosciences, Cat. No. 17-0405-01) was rinsed with 50ml of start buffer (20mM Sodium Phosphate at pH 7) and then the dialysed TCSN was applied (1ml/min). The column was washed with 50ml of start buffer to remove unbound proteins Bound antibody was eluted using 20-50ml of elution buffer (0.1M Glycine pH 2.7) at a flow rate of 1ml/min, collecting 1ml fractions into 80µl of 1M Tris, until Bradford test was negative. The column was then washed with 50ml of start buffer and stored in 20% ethanol at 4°C.

The protein concentration (mg/ml) of the eluted fractions was determined using an Eppendorf *Bio*photometer (Eppendorf GMBH, Germany). This determined the protein concentration at OD_{280} and an absorbance of 1 was assumed to be equivalent to 1mg/ml. All fractions above 0.2mg/ml were pooled and dialysed against 1 x PBS as described above.

2.3.7.4 Production of Vivaspin columns

Vivapure Epoxy protein coupling mini spin columns (Vivascience Cat. No. VS-PC01EPPC) were used following manufacturers' instructions. Briefly, 2ml of Buffer A (2x) was diluted with 2ml of distilled water per column prepared. The protein sample was concentrated to 1-2mg/ml and 200µl of the protein sample was added to 200µl of buffer A (coupling buffer). The epoxy membrane was equilibrated by centrifugation (2,000 x g for 5min) with 400µl of 1:1 diluted buffer A and the flow through was discarded. The 400µl of coupling solution was loaded onto the epoxy membrane and centrifuged at 400 x g for 2min. The flow through was re-pipetted onto the membrane and kept at room temperature for 3h. Following this incubation

the coupling solution was re-centrifuged through the membrane at 2,000 x g for 5min. To wash the column 400µl of diluted buffer A was centrifuged through the column, followed by 400µl of buffer B, followed by 400µl of diluted buffer A all at 2,000 x g for 5min. All the flow throughs were discarded and the columns were then ready to test for conditions of affinity purification.

2.3.7.5 Production of CnBr coupled Immunoaffinity Column

Antibodies were dialysed against 2.5 litres of coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl at pH 8.3) as described above (2.3.7.3) and the Eppendorf *Bio*photometer was used to check the concentration at all stages.

The Vivacell 70 concentrator (Vivascience Cat. No. VS6022) and 5 bar of pressure were used to concentrate antibodies to 5mg/ml. For every 5ml of purified antibody at 5mg/ml, 1g of Cyanogen Bromide Sepharose^{TM}4B (Amersham Biosciences, Cat. No. 17-0430-01) was required. The appropriate amount was weighed into a 50ml falcon tube and hydrated with 1mM HCl. A scintered glass filter was prepared by washing with 20% ethanol and then with distilled water. The sepharose was added to the sintered glass filter and for every gram of sepharose 200ml of 1mM HCl was run through the filter.

The sepharose and antibody were mixed in a 50ml falcon tube for 1h at room temperature, rotating end over end. The excess ligand was washed away with 5 gel volumes of coupling buffer, by mixing and centrifuging at 1,400 x g/5min (IEC Centra-4R). The supernatant was removed, and stored to check the antibody concentration and therefore the amount (mg) of antibodies not bound. 20ml of 0.1M Tris HCl pH 8 was added to the sepharose, mixed and then left to stand for 2h. The supernatant was removed and stored to check the antibody concentration.

The sepharose was washed with 3 cycles of alternating pH (0.1M Acetate Buffer with 0.5M NaCl at pH 4 and 0.1M Tris HCl Buffer with 0.5M NaCl at pH 8), using 5 column volumes of each buffer for each wash. The washes were checked for unbound antibody. The column was stored in storage buffer (50mM Tris HCl, 150mM NaCl, 0.02% azide at pH 8).

2.3.7.6 Conditions for Immunoaffinity Chromatography

A P1 pump was used to maintain the flow rate at 1ml/min. To equilibrate the column 5 x column volumes of binding buffer (50mM Tris pH 8) was used. The concentrated HIV-2 gp120 containing TCSN (desalted into binding buffer) was then passed over the column three times, followed by 1 column volume of binding buffer. The column was washed with 5 x column volumes of wash buffer (50mM Phosphate Buffer 10mM NaCl pH 5.5) and bound HIV-2 gp120 was eluted using elution buffer (50mM glycine 10mM NaCl pH 2.5) with 1ml fractions being collected into 1:20 volume (50µl) of collection buffer (1M Tris pH 9.0), until fractions were Bradford negative. The column was then stored in storage buffer (as above: 2.3.7.5). All column 'flowthrough' fractions were stored for subsequent analysis.

2.3.8 Size Exclusion Chromatography

The concentrated HIV-2 gp120 containing TCSN was filtered through a 0.45µm filter. All buffers (Superose 6 - 1M NaCl, 50mM HEPES at pH 6.5, Superdex 200 - 300mM NaCl, 50mM Tris at pH 8) were filtered (0.45µm Whatman, Cat. No. 7184-004) and degassed before use on the FPLC. The pumps were washed with distilled water containing 0.02% sodium azide. Following this there was a pump wash using the appropriate FPLC buffer. The loop was washed with 5 loop volumes (1ml) of the appropriate FPLC buffer. Following this the column was connected and washed with 2 column volumes (50ml) of FPLC buffer. A flow rate of 0.25ml/min was required for Superose 6 and 0.5ml/min for Superdex 200 throughout the experiment.

100µl of TCSN (previously purified over a GNA lectin affinity column followed by ARP 3085 immunoaffinity column) was injected into a 200µl volume loop. The protein was injected over the column followed by 5 loop volumes of buffer to ensure that all the protein entered the column. One column volume (24ml) of 0.5ml fractions was collected for analysis.

The column was cleaned by passing over 2 column volumes of water containing 0.02% sodium azide. The column was disconnected, the loop washed as above and the pumps were washed with 20% ethanol for storage.

2.4 HIV-2_{ROD} gp120 characterisation

Having established purification protocols (see results) a range of techniques were applied to ascertain the properties of the glycoprotein before starting crystallisation trials.

2.4.1 Wavelength Scan

A wavelength scan of pure protein was used to make a more accurate determination of concentration using a Beckman (USA) Du® 640 Spectrophotometer. 80µl of buffer was used as a blank across the spectrum of 240nm-320nm. Following this 80µl of protein was assessed at these wavelengths and the reading at 280nm used with the extinction coefficient ($\varepsilon(280) = 183{,}325\ M^{-1}cm^{-1}$) of the HIV-2 gp120 to determine concentration.

2.4.2 Native Gels

To help determine the oligomeric state of the HIV-2 gp120 envelope glycoprotein, PAGE was run without β mercaptoethanol and SDS (See Appendix Method 4). Samples were added to native gel sample loading buffer (5x) (250mM Tris pH 7, 0.5% bromophenol blue, 50% glycerol) and the molecular weight marker, Native Gel Protein Mixture (Amersham Biosciences, Cat. No. 17044501), was added at a ratio of 1:1 with the loading buffer (2 lanes are required when western blotting – this was to produce one marker lane for staining prior to electroblotting of the remaining lanes). Samples were not heat denatured before loading onto the gel and running as for SDS PAGE (section 2.2.5). However a different running buffer was required containing 250mM Tris and 2M Glycine/litre. For western blotting one marker lane was removed from the gel before electroblotting and simply blue stained (see section 2.4.3.2). Following detection using ECL the blot was stained using Coomassie Blue (see section 2.4.3.3) to detect some of the markers.

2.4.3 Protein Staining

Protein staining methods were used to assess the purity of gp120 at various stages of the purification process

2.4.3.1 Silver Stain

This sensitive method was used to determine the purity of HIV-2 gp120 in the final stages of purification. After electrophoresis, gels were stained using Pierce GelCode® Silver Stain Kit II (Cat. No. 24612) following manufacturer's protocols. Briefly, the gel was washed twice for 5min with ultrapure water and then fixed using a solution of 30% ethanol:10% acetic acid for 15min at room temperature, followed by another 15min incubation in the same buffer. After fixing the gel was washed twice each for 5min in 10% ethanol and, finally, ultrapure water.

Sensitiser Working solution was prepared (0.1ml Sensitiser with 50ml water) and used to sensitise the gel for 1min, followed by two 1min washes with water. Stain Working solution (1ml Enhancer with 50ml Stain) was then added to the gel for 30min, followed by washing with ultrapure water for a total of 40s changing the water once.

Developer Working solution (1ml Enhancer with 50ml Developer), was used to develop the gel for 2-3min until bands appeared. Development was stopped by immersing the gel in 5% acetic acid for 10min. The detection limit of this procedure was approximately 1ng of protein.

2.4.3.2 Simply Blue Stain

The simply blue stain (see section 2.6.13.2 for recipe) was used to assess purity of HIV-2 gp120 protein suitable for crystal trials. Following electrophoresis, denatured reduced gels were washed twice for 5min in ultrapure water before adding the simply blue stain which was left to stain for 30min to overnight. The gel was destained in water, to reveal protein bands with a detection limit of 5-10µg.

2.4.3.3 Coomassie Blue Stain

Following blotting of a native PAGE gel, coomassie blue stain (see section 2.6.13.1) was added to the blot for 10s, to detect the Protein mixture markers. After decanting the stain, the blot was destained using a solution of 45% methanol:10% acetic acid for 10min changing the destain regularly, to reveal the protein bands.

2.4.3.4 Glycoprotein Stain

Following treatment with PNGase F (Roche, Cat. No. 11 365 185 001) HIV-2 gp120 was checked for the presence of sugars using a glycoprotein stain. After electrophoresis, gels were stained using a Pierce GelCode® Glycoprotein Staining Kit (Cat. No. 24562) following manufacturer's protocols. Briefly, the gel was fixed by completely immersing it in 100ml of 50% methanol for 30min. The gel was washed twice by gently agitating with 100ml aliquots of 3% acetic acid for 10min.

The gel was then transferred to 25ml of Oxidising Solution (Reconstituted powder with 250ml of 3% Acetic Acid) and then gently agitated for 15min. The gel was washed three times by gently agitating with 100ml aliquots of 3% acetic acid for 5min.

The gel was then transferred to 25ml of GelCode® Glycoprotein Staining Reagent and gently agitated for 15min. Following this the gel was immersed in 25ml of Reducing Solution (reconstitute powder with 250ml of ultrapure water) and gently agitated for 5min. Finally, the gel was washed extensively with 3% acetic acid and then with ultrapure water. Glycoproteins appeared as magenta bands and the gel was stored in 3% acetic acid. The detection limit of this procedure was approximately 5-10µg of glycoprotein.

2.4.3.5 Gel Drying

Following staining, gels were preserved by immersing in gel drying solution (20% Methanol; 3% Glycerol) for a minimum of 30min. Novex gel drying stands with DryEase™ Mini Cellophane (Invitrogen, Cat. No. NC2380) were then used to dry the gels overnight at room temperature.

2.4.3.6 2D Electrophoresis

15µl of sample was added to 110µl of urea-thiourea buffer [5M urea (PlusOne, GE Healthcare, Cat. No. US75826-5KG), 2M thiourea (Fluka, Cat. No. 88810), 2% CHAPS (Sigma, Cat. No. C3023), 2% SB 3-10 (Fluka, Cat. No. 30694), 65mM DTT (SigmaUltra, Cat. No. D5545), 20mM Tris pH 9.5, 0.1mM EDTA].

The 125µl sample was mixed with a grain of bromophenol blue and immobilised pH gradient (IPG) buffer (GE Healthcare, Cat. No. 17-6001-12) to make a final IPG buffer concentration of 0.8% (v/v). It was then centrifuged in a microcon filter tube (0.22µm pore; Millipore, UK) at 9000 x g for 3min.

A pH 3-10, 7cm IPG strip (GE Healthcare, Cat. No. 17-6001-12) was rehydrated overnight with the sample in an Immobiline DryStrip Reswelling Tray (GE Healthcare, UK). During rehydration, the strip was overlaid with IPG cover fluid (GE Healthcare, Cat. No. 17-1335-01) and the tray covered with Saran Wrap® (The Dow Chemical Company, USA).

IEF was performed the following day using the Multiphor II system (GE Healthcare, UK). The Immobiline DryStrip tray (GE Healthcare, UK) and DryStrip aligner (GE Healthcare, UK) were correctly positioned and then the rehydrated IPG strips were positioned gel-side-up on the aligner. The acidic ends of the strips were near the anode whilst the basic ends were near the cathode. Two electrode strips (GE Healthcare, Cat. No. 18-1004-40) were moistened with ddH_2O and placed over the acidic and basic ends of the strips, perpendicular to the strips. The anode bar (GE Healthcare, UK) was fitted over the electrode strip at the acidic end and the cathode bar (GE Heatlthcare, UK) was fitted over the electrode strip at the basic end. The tray was filled with 230ml of IPG cover fluid. A MultiTemp III thermostatic circulator (GE Healthcare, UK) was used to set the temperature to 20°C. An EPS 3501XL power supply (GE Healthcare, UK) was programmed to run 7 phases in the order shown in Table 2.6. When the IEF was complete, the second dimension was either performed straight away, or the IPG strips were stored at –80 °C until required.

Phase	Voltage (V)	Current (mA)	Resistance (Ohms)	Time (h:min)	Volthours
1	150	2	5	0:30	75
2	300	2	5	2:30	750
3	700	2	5	0:30	350
4	1500	2	5	0:30	750
5	2000	2	5	0:30	1000
6	3000	2	5	0:30	1500
7	3500	2	5	16:00	56000

Table 2.6 IEF programme

The second dimension run was carried out using a Mini Protean II gel electrophoresis apparatus (Bio-Rad, UK). A 10% acrylamide gel was made using 1.5mm gel spacers and the resolving gel components described in Appendix Method 2. The caster was filled with the resolving gel solution to 5-10mm below the top and the gel overlaid with water-saturated butanol. The gel was allowed to polymerise for 20min.

While the gel was setting, the IPG strip was equilibrated in the reswelling tray with gentle shaking. The strip was bathed in 3ml of equilibration buffer (50mM Tris pH 8.8, 6M urea, 30% (v/v) glycerol, 2% (v/v) SDS) containing 2% (w/v) DTT (SigmaUltra, Sigma-Aldrich, UK) for 15min, followed by another 15min in equilibration buffer with 2.5% (w/v) iodoacetamide (SigmaUltra, Sigma-Aldrich, UK) and a grain of bromophenol blue.

When the resolving gel was set, its surface was rinsed with double distilled water (DDW). The equilibrated IPG strip was placed on the surface of the gel, avoiding air bubbles between the two gel surfaces. The strip was set in place with stacking gel (see Appendix Method 2). See Blue (Invitrogen, Cat. No. LC5925) Molecular weight markers were loaded on a separate gel. The gel was run in SDS electrophoresis buffer at a constant voltage of 200V for 80min, followed by electroblotting at 15V for 1h.

2.4.4 Dynamic Light Scattering

Dynamic Light Scattering was used to determine the polydispersity (a measure of the distribution of molecular weights in a given sample) of gp120 samples in the final stages of purification. Proteins with polydispersity values of less than 30 are considered suitable for crystallisation trials and estimates of molecular weights, based on the hydrodynamic radius measured, can be made (D'Arcy, 1994; Ferre-D'Amare and Burley, 1997).

This was performed using a Temperature Controlled Microsampler and a Dyna Pro Molecular Sizing Instrument (Protein Solutions). The cuvette used to take measurements was washed at the beginning and end of an experiment. This involved washing the cuvette with filtered water, followed by 2% Hellmanex® II (Hellma GmbH & Co.), followed by 100% ethanol and drying with canned air. Since HIV-2 gp120 is a large protein, a concentration of approximately 0.5mg/ml was required to yield an accurate reading. Suitably concentrated samples were clarified by centrifugation at 16,100 x g/30min and 20µl aliquots were added to the cuvette. All experimental readings were taken at 18°C and the average of 10 readings was used to generate the result. Data, was analysed, saved and printed using the software provided (Dynamics Software).

2.4.5 Enzyme Based ImmunoSorbant Assay (ELISA)

ELISA was used for both quantifying the amount of HIV-2 gp120 recovered at each stage during the purification process (see results) and to determine whether the HIV-2 gp120 bound CD4 (see results).

The antigen was diluted in sensitising buffer (75mM $NaHCO_3$, 25mM Na_2CO_3 made up to 100ml with DDW (pH 9.5)) and then the antigen (either 0.25µg of CD4 for HIV-2 gp120 binding activity or HIV-2 gp120 dilution series for quantification see results section 3.2.5.1) was added to the ELISA plate at 50µl/well. The plate was incubated overnight at 4°C with gentle shaking.

For HIV-2 gp120 quantification, the plate was washed in washing buffer (150mM NaCl, 0.1% Tween 20 made up to 2L with DDW) four times using an automated Wellwash 4 (Denley). This wash procedure was used in between each incubation step. 50µl of blocking buffer (1.0g Marvel made to 100ml with PBS) was added to each well and incubated (covered) at room temperature for 1h. Primary antibody was prepared by dilution in buffer (0.5g Marvel, 50µl Tween 20 made to 100ml with PBS) to a concentration of 1µg/100µl, then 50µl was added to each well and incubated at room temperature for 1h. 50µl of secondary antibody (anti-mouse IgG Horse Radish peroxidase conjugate (Promega, Cat. No. W402B 1mg/ml) used at 1/1000 in diluting buffer) was added to each well and incubated at room temperature for 30min. After washing 50µl of substrate (Sigma, Cat. No. P-9187), made up fresh, was added to each well and incubated at room temperature for 30min. Incubation took place in the dark, and the reaction was checked every 5min. The plates were read using a Thermo Labsystems Multiskan Ascent/Labsystems Multiskan® Bichromatic machines at 450nm.

For CD4 binding determination experiments an extra step was added following blocking of the wells, a purified HIV-2 gp120 (39.5 8) dilution series was added to the wells and incubated at room temperature for 1h followed by washes as above and the primary antibody addition.

2.4.6 Surface Plasmon Resonance (BIAcore)

BIAcore was used to determine functionality of the HIV-2 gp120 protein produced, in terms of it's affinity for CD4 and conformation specific antibodies. All experiments were performed at 37°C using a BIAcore®2000 system with BIACORE 200 control software 3.1.1 (BIAcore Inc., Uppsala, Sweden).

Antibody binding experiments required a running buffer containing 50mM Tris, 10mM sodium chloride at pH 8 (which were the same conditions used for binding protein in immunoaffinity columns) while CD4 binding required 10mM HEPES, 150mM NaCl and 0.005% Tween 20 (HBS-P) pH 7.4, which is a standard buffer used in BIAcore experiments.

HIV-2 gp120 partially purified by immunoaffinity chromatography (ARP 3032) was bound to the chip for antibody binding experiments and the negative control used was a blank surface. For CD4 binding experiments, CD4 was bound to the chip and, due to the stickiness of HIV-2 gp120, BSA was added to the blank chip to block binding sites. These proteins were covalently attached to a cell on a research grade CM5 chip (BIAcore), using the amine coupling kit according to manufacturers' instructions. Briefly this included determination of the correct pH for binding. A flow rate of 10µl/min was used. 50µg/ml (100µl) of protein adjusted to pH 5.5 was injected over the surface of the chip and the response units (RU) were measured, this was then repeated with protein at pH's 5, 4.5 and 4. The pH that gave the highest number of RU (> 10,000 RU) without denaturing the protein was the condition chosen. For both experiments protein adjusted to a pH of 5 caused the highest response to injection. The chip surface was then activated following manufacturers' instructions, the protein bound and subsequent to this any unbound sites on the chip were blocked using ethanolamine.

Before affinity measurements were taken the regeneration conditions were determined. Protein was injected until a plateau was reached, the RU were measured and then a regeneration condition was injected. This was repeated until a regeneration condition removed >90% of the bound protein and when protein was rebound this 90% returned. The regeneration conditions for the antibody binding experiments were an injection of 5µl of 1.5M Glycine (pH 2.5) followed by a 300s wait, and for the CD4 binding experiments an injection of 6µl of 50mM HCl followed by an EXTRACLEAN step (command name) with HBS-P to remove any excess acid for the loop and the Integrated µ-Fluidic Cartridge (IFC) followed by a 300s wait step before a subsequent run.

Also the time to reach equilibrium is required and this was determined by injecting a large volume of protein at a chosen flow rate. 20µl of antibodies were passed over the chip at a flow rate of 10µl/min to achieve equilibrium whereas 5µl of HIV-2 gp120 injected at 2µl/min was required when binding CD4.

To estimate a K_D a series of five fold dilutions was injected and approximate calculations using the BIAevaluation version 3.2 software were undertaken. The estimated K_D for ARP 3083 (Rat monoclonal antibody recognises an epitope in C1 of HIV-2 Env) binding to ARP 3032 purified HIV-2 gp120 was 9nM and for HIV-2 binding to CD4 it was 24μM.

To more accurately determine K_D values required a dilution series of the antibodies of HIV-2 gp120 to be made, starting at 10-100 x the estimated K_D (see above), containing at least 10 two-fold dilutions. The protein was injected over the chip, the volume and flow rate of which was determined by the equilibrium experiment described above. This was followed by the regeneration as determined previously.

The data were analysed by the BIAevaluation version 3.2 software and, after subtraction of reference cell data, fitted to a 1:1 binding model.

2.4.7 Circular dichroism spectroscopy

Others have shown experimentally that the HIV-1 SU has secondary structure of 22% α-helix and 38% β-strand respectively (Hansen et al., 1996). The circular dichroism (CD) spectra of proteins in the far-UV region of the spectrum (190-250nm) derive almost entirely from the amide chromophore, with (usually) small contributions from aromatic residues. These spectra reflect protein secondary structure and the far-UV spectrum of a protein can be used to estimate its secondary structure content (Sreerama and Woody, 2000).

CD measurements were performed using a Jasco J-175 spectropolarimeter. Far-UV CD spectra were recorded at 20°C using 1mm fused silica cuvettes with a HIV-2 gp120 concentration of 9.5μM. For each spectrum, four scans were averaged and the baseline subtracted. CD intensities are presented as the CD absorption coefficient calculated on a mean residue weight (MRW – protein mean weight/number of residues) basis.

2.4.8 Fluorescence spectroscopy

Fluorescence spectroscopy was used to determine if the protein was folded. The intrinsic fluorescence of proteins derives principally from the aromatic residues Tyr and Trp. Both the quantum yield (fluorescence intensity) and the wavelength of maximum emission can be affected by changes in the environment of the side chain. In the case of Trp, for example, the position of the emission maximum is often taken as a measure of the extent to which the Trp is buried in the protein interior. The emission maximum of Trp can vary from ~355nm, when Trp is exposed to water, to ~322nm, when it is totally buried. The fluorescence signal of HIV-2 gp120 was determined from the contribution of its 25 Trp residues.

Fluorescence emission spectra were recorded using a SPEX FluoroMax fluorimeter, with excitation at 280nm (bandwidth 1.7nm) and emission scanned from 290 to 400nm (bandwidth 5nm).

2.4.9 Analytical Ultracentrifugation (AUC)

AUC was used to establish HIV-2_{ROD} gp120's solution molecular weight and hence its oligomeric state.

2.4.9.1 Equilibrium Analytical Ultracentrifugation Measurements

Equilibrium analytical ultracentrifuge measurements were made with a Beckman XL-A analytical ultracentrifuge (Beckman Coulter, Fullerton, CA) using a 4-position AnTi60 rotor. Each cell had a 6-channel carbon-epon centrepiece with two quartz windows giving an optical path length of 1.2cm and each cell was scanned stepwise (0.003cm steps) at a wavelength of 280nm and temperature of 20°C. Absorbance was monitored relative to buffer. The program Sednterp (http://www.cauma.uthscsa.edu/software) (Cole and Hansen, 1999) was used, with HIV-2 gp120's amino acid composition and carbohydrate content, to calculate the proteins partial specific volume at 20°C. This gave a value of 0.6880 and an estimated monomeric molecular weight of hexa-His tagged HIV-2 gp120 of 126.5KDa. The solvent density (1M NaCl 50mM Hepes pH 6.5) was calculated to be 1.04g/ml at 20°C. Solutions of 1.4μM, 2.2μM and 3.4μM HIV-2_{ROD} gp120 (these correspond to optical densities of 0.24, 0.36 and 0.57 at 280nm respectively) were

centrifuged at rotor speeds of 2016 x g/28h (readings taken at 24h, 26h and 28h), 4536 x g/18h (readings taken at 14h, 16h and 18h) and 8064 x g/18h (readings taken at 14h, 16h and 18h). All data sets at each speed were overlayed to show equilibrium had been achieved. The nine data sets (three concentrations, three speeds) where then fitted to the appropriate equation for a single species using Beckman Optima XL-A/XL-1 data analysis software, version 4.0.

2.4.9.2 Velocity Analytical Ultracentrifuge Measurements

Sedimentation velocity experiments were conducted with a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter, Fullerton, CA) equipped with absorbance optics. For sedimentation velocity experiments, a four hole (AnTi60) rotor was used. The rotor temperature was equilibrated at 20°C for 1-1.5h in the vacuum chamber prior to the start of each run. Quartz windows were used (absorbance optics) with Epon double-sector centrepieces giving an optical path length of 1.2cm. The experiment was conducted at 20°C and rotor speed of 129,024 x g. The cell was scanned stepwise (0.01cm) at a wavelength of 280nm. The sample analysed was in a 1M NaCl, 50mM HEPES solution at pH 6.5 and had an optical density of 1 (equivalent to 0.5mg/ml) Absorbance and interference scans were collected at time intervals of 5min and 200 scans were used in the analysis. Initially a simple analysis carried out by the transport method (using Beckmans Software) and a sedimentation coefficient is determined. Further analysis of the sedimentation velocity profiles of the HIV-2 gp120, in terms of the differential sedimentation coefficient distribution c(s) was performed with the program SEDFIT (Schuck, 2000).

2.4.10 Negative Staining Electron Microscopy

Electron microscopy, carried out by Lesley Calder (Virology, NIMR), was used to visualise HIV-2 gp120's oligomeric state. Stock solutions of both HIV-2_{ROD} gp120 and monoclonal antibodies were kept at between 0.2-0.5mg/ml. Complexes of monoclonal antibodies and HIV-2 gp120 were allowed to form by mixing at room temperature of individual concentrations of 0.1mg/ml (diluted in PBS) and leaving for two hours before adding to the grid. Gp120 in isolation and as complexes was absorbed onto carbon coated grids and negatively stained with 1% sodium

silicotungstate (pH 7.5). A JEOL 1200EX electron microscope operated at 100KV was used to view the grids. Micrographs were taken under minimum dose conditions at a magnification of 30,000 x.

2.5 Crystal Trials

Having established the purity and functionality (CD4 binding and binding of conformation-dependant monoclonal antibodies) I proceeded to set up crystallisation trials.

2.5.1 Manual Setup

Glass cover slides were cleaned with 100% ethanol. 7 release compound (Dow Corning® USA, Cat. No. 1966944-0799) was used to grease round the edge of the wells. 0.5ml of mother liquor (The particular condition being assayed for crystal formation) was pipetted into the well (24 well). 0.25µl (1.3mg/ml) of protein was pipetted onto the cover slip (being careful to avoid bubbles) and then 0.25µl of mother liquor was added to the protein on the cover slip. The cover slip was quickly placed over the well, with the drop facing the well. The large end of a 1ml pipette tip was used to press down the cover slip. This was repeated with different mother liquors. The screen (A screen contains multiple mother liquors containing different components and at different pH's and molarities) utilised was the PS Screen (Emerald Biosystems, Cat. No. EBS-TPS-P1) (Majeed et al., 2003). Following setup of drops the trays were placed at 18°C to store. The drops were monitored by microscopy for crystal formation.

2.5.2 Robotic Setup

The robots used were Oryx6 and the XYZV Plateloader, from Douglas Instruments. They were both controlled by a computer and manipulated using the Oryx6 software package. They were used to set up sitting drops in CrystalClear strips with platforms. 200nl of sample (1.3mg/ml) and 100nl of mother liquor was used per drop set up. The screens utilised were The Classics Suite (Nextal Biotechnologies), Macrosol™ MDI-22 (Molecular Dimensions Ltd) and Stura Footprint Screens MDI-20 (Molecular Dimensions Ltd). Following setup of drops,

strips were placed at 18°C to store, and drops were monitored for crystal formation by microscopy.

2.6 Equipment and Materials

2.6.1 Major Equipment

- ÄKTA FPLC (Amersham Biosciences)
- Balance BD202 (Mettler Toledo).
- Benchtop spinmixer (GallenKamp).
- BIACORE 2000 (BIAcore)
- Blood tube rotator SB1 (Stuart Scientific)
- Centrifuges: IEC Centra-4R (Damon, UK); Eppendorf Centrifuge 5415R + 5415C (Eppendorf GMBH, Germany); Beckman J6-HC Centrifuge (Beckman); Hermle Z400K (Hermle); Beckman Optima XL-A Analytical Ultracentrifuge (Beckman), MicroCentaur (Sanyo).
- Class II Microbiological Safety Cabinet (Medical Air Technology Ltd, Manchester)
- Crystal Robot: XYZV Plateloader (Douglas Instruments); Oryx6 (Douglas Instruments)
- DNA electrophoresis apparatus: made in-house.
- Dynamic Light Scattering: Temperature Controlled Microsampler (Protein Solutions); Dyna Pro Molecular Sizing Instrument (Protein Solutions); Dynamics Software.
- Eppendorf Biophotometer (Eppendorf GMBH, Germany).
- Filter unit (Nalgene)
- FPM 2100 X-ray Film Processor (Fuji, Japan).
- Freezers: K40-8 Kelvinator -20°C freezer; Bauknecht -20°C freezer; Lab Impex Research -70°C.
- Incubators: MCO-17A1 CO_2 Incubator (Sanyo); Cabinet Incubator (GallenKamp); G24 Environmental Incubator Shaker (New Brunswick Scientific); Orbital Incubator S150 (Stuart Scientific); Memmert.
- IS550 Imager (Kodak).
- JASCO J-715 Spectropolarimeter; PS-150J Power Supply (JASCO)

- Magnetic Stirrer/hotplate MR3001 (Heidolph).
- MegaBACE Capillary Sequencer (Amersham Biosciences, UK).
- Microscopes: Nikon Eclipse TS100 (Nikon, Japan); Nikon SMZ1000 (Nikon, Japan) with Photonic PL3000; JEOL 1200EX electron microscope
- Mini PROTEAN® 3 Cell (BIO RAD)
- Multimix MM1 (Luckham)
- Peristaltic Pump P-1 (Pharmacia)
- pH meter 240 (Corning)
- Plate Readers: Labsystems Multiskan® Bichromatic (Labsystems); Multiskan Ascent Plate Reader (Thermo Labsystems, UK).
- Pipettes: Pipetteman (P2, P10, P20, P200 and P1000)(Gilson); Multipipette® Plus (Eppendorf GMBH, Germany); Multichannel pipette (Labsystems); Microliter Syringe (Hamilton, Switerland); Microman (P50, P250)(Gilson); Pipetteboy acu (Integra Biosciences).
- Power Packs: Gene Power Supply GPS 200/400 (Pharmacia); Powerpac 200 power pack (BioRad); PowerPac Basic (BioRad).
- Spectrophotometer Du® 640 (Beckman)
- Spex FluoroMax™ (Jobin Yvon)
- Thermal Cyclers: PTC-100 (MJ Research Inc.); PTC-200 (MJ Research Inc.).
- Trans-Blot SD semidry transfer apparatus (BioRad).
- Transmission Electron Microscope JEOL 1200EX
- UV transilluminator (BDH).
- Water Baths (Grant Instruments Ltd, UK).
- Wellwash 4 (Denley)
- Xcell SureLock™ protein electrophoresis apparatus (Novex).

2.6.2 Laboratory Consumables

- O.1µm Whatman Inorganic membrane filter (Whatman)
- 0.2ml Thin walled PCR tubes (The Perkin Elmer Corporation, USA)
- 0.5ml flip-top tubes (Treff Lab, Switzerland)
- 1.0mm thick SuperFrost glass slides (BDH)
- 1.5ml flip-top tubes (Treff Lab, Switzerland).

- 1.5ml Graduated w/o ribs screw tubes (Scientific Specialities Inc, U.S.A.)
- Filtered pipette tips 10µl, 20µl, 250µl and 1000µl. (Rainin, UK)
- Disposable Pipettes (1ml, 5ml, 10ml, 25ml and 50ml)(Costar, UK)
- 13mm round coverslips (BDH)
- 14ml Falcon tubes (Becton Dickenson)
- 15ml Centrifuge tubes (Cornig)
- 2.5ml Screw top tubes (Sarstedt)
- 20ml Universal tubes (Sterilin)
- 2ml Cryotubes (Nunc)
- 50ml Centrifuge tubes, skirted (Corning)
- 6/12/24 well plates (Nunc)
- 60mm Tissue culture dishes (Orange)
- 48/96 well flat-bottomed plates (Corning Incorporated, Costar)
- Bijoux (Sterilin)
- Cellulose Nitrate Membrane Filters 0.45µm (Whatman)
- Combitips Plus 2.5ml and 5.0ml (Eppendorf GMBH, Germany)
- CrystalClear Strips with platform (Douglas Instruments)
- Crystal Plates 24 well (Hampton Research)
- Cuvettes UVette (Eppendorf GMBH, Germany)
- Dialysis Tubing, 12kDa cut off (SIGMA)
- Disposable sterile scalpels no.11 (Swann-Morton, UK)
- Hybond^{TM}C Extra nitrocellulose membrane (Amersham Biosciences, UK)
- Kodak MXB film (Kodak)
- Minisart filters 0.45µm and 0.20µm (Sartorius)
- Natural Pipette Tips 0.1-10µl, 1-200µl and 1000µl (Starlab GMBH)
- Pre-cast 15 well 4-12% Bis-Tris polyacrylamide gells (Novex)
- Siliconized Glass Circle Cover Slides, 22mm (Hampton Research)
- Syringes 5,10,20 and 50ml (Becton Dickenson Labware, UK)
- Tissue culture flasks T25cm^2, T80cm^2, T175cm^2 and triple layered T175cm^2 (Nunc)
- Ultrafiltration Membranes 10KDa and 30KDa (Millipore)

- Ultrafree-MC Centrifugal Filter Devices (Millipore)
- Vivacell 70 30KDa (VS6022, Vivascience)
- Vivaspin 500 3KDa, 10KDa, 30KDa (VS0191, VS0102, VS0121 Vivascience)
- Vivaspin 6ml Concentrator 10KDa, 30KDa (VS0601,VS0621, Vivascience)

2.6.3 Complete Kits

- Applied Biosystems Big Dye terminator v1.1 cycle sequencing kits (Cat. No. 4337450 and 4337036, Applied Biosystems, UK)
- Effectene Transfection Kit (Cat. No. 301425, Qiagen)
- Lipofectamine 2000™ Transfection Kit (Cat. No. 11668-027, Invitrogen)
- Macrosol™ MDI-22 (Molecular Dimensions Ltd)
- Profection Transfection Kit (Cat. No. E1200, Promega)
- Qiagen Midiprep kit (Cat. No. 12143, Qiagen, UK)
- Rapid DNA Ligation Kit (Cat. No. 1 635 379, Roche)
- Rapid Pure Miniprep kit (Cat. No. 2070-400, Qbiogene, USA)
- Pierce GelCode® Silver Stain Kit II (Cat. No. 24612)
- Pierce GelCode® Glycoprotein Staining Kit (Cat. No. 24562)
- PS Screen (Emerald Biosystems Cat. No. EBS-TPS-P1) (Majeed et al., 2003)
- Stura Footprint Screens MDI-20 (Molecular Dimensions Ltd)
- Superfect Transfection Kit (Cat. No. 301305, Qiagen)
- The Classics Suite (Nextal Biotechnologies)

2.6.4 DNA Plasmid Vectors

- pEE14tPAgp1203 (Celltech now LONZA Biologics)

2.6.5 Enzymes

- Calf Intestinal Alkaline Phosphatase (CIAP)(Invitrogen Life Technologies, UK)
- *pfu* polymerase (Stratagene).
- PNGase F (Roche)
- Restriction enzymes and buffers (Roche, UK)

2.6.6 Standards and Controls

- λ DNA BstE II digested ladder, 50µg/ml (NEB, Cat. No. 301-4)
- 2 Domain CD4 *E.coli* (NIH, Cat. No. 7356) (Garlick et al., 1990)
- Baculovirus produced HIV-2 gp105 (CFAR, EVA 621)
- IEF Marker pH 3-10 (Invitrogen, Cat. No. 39212-01)
- Native Gels Protein Mixture (Amersham Biosciences, Cat. No. 17044501)
- SeeBlue® Plus 2 protein marker (Invitrogen, UK, Cat. No. LC5925)
- Prestained SDS-PAGE Standards – Broad Range (BIO-RAD, Cat. No. 161-0318)

2.6.7 Commercial Reagents

All general chemicals were analytical or ultrapure grade, supplied by Sigma-Aldrich, UK or BDH Ltd., UK unless otherwise stated.

- 2% Hellmanex® II (Hellma GmbH & Co.)
- 30% Acrylamide/Bis Solution 37.5:1 (Bio-Rad Laboratories, California, USA)
- 96% Ethanol (Fisher Scientific, UK)
- Acetic Acid glacial (Fisher Scientific,UK)
- Ammonium Persulfate (Sigma, UK)
- Ammonium Sulphate (BDH)
- β-Mercaptoethanol (Sigma-Aldrich, UK)
- Bovine serum Albumin (BSA) (Sigma-Aldrich, UK)
- Bradford Reagent (BioRad)
- Calcium Chloride (Sigma, UK)
- Citifluor (UKC Chem. Lab.)
- Complete, protease inhibitor cocktail tablets (Roche, Germany)
- DAPI - 4'6-Diamidino-2-phenylindole dihydrochloride (Sigma D 9542)
- Dow Corning® 7 Release Compound (Dow Corning Corporation, USA)
- Electro-chemiluminescence (ECL) development reagent (Amersham Pharmacia Biotech)
- Ethidium Bromide tablets (Amresco, Ohio, USA), used at 1µg/ml working concentration.
- Glycerol (BDH)
- Glycine (BDH)

- GS Supplement (JRH Biosciences)
- HEPES (Sigma, UK)
- Hydrochloric Acid (11.6N) (BDH)
- Interleukin 6 (IL-6) (Roche)
- Magnesium Chloride (Sigma, UK)
- Methanol (Fisher Scientific, UK)
- Methyl α-D-mannopyranoside, minimum 99% GC (Sigma, UK)
- Milk Powder (Marvel)
- Nonidet P40 (BDH)
- NuPAGE® MOPS SDS buffer (Invitrogen)
- NuPAGE™ LDS Sample Buffer 4x (Invitrogen)
- Paraformaldehyde (BDH)
- Penicillin/Streptomycin (P/S) (100units/100µg /ml, Sigma)
- Phenol/chloroform (Amresco, Ohio, USA)
- Polyethylene glycol (Sigma, UK)
- Polyoxyethelenesorbitan monolaurate (Tween20) (Sigma-Aldrich, UK)
- Protogel Buffer (National Diagnostics)
- Propan-2-ol (Fisher Scientific, UK)
- Romil SpS™ water (Romil Ltd., UK) autoclaved for reconstitution or elution of DNA
- Sodium Azide, minimum 99.5% (Sigma, UK)
- Sodium Chloride (Fisher Scientific, UK)
- Sodium dodecyl sulphate (SDS) (Bio-Rad)
- TEMED (N,N,N',N'-Tetramethylethylenediamine)(Sigma, UK)
- Trichloroacetic Acid (Fisher Scientific,UK)
- Tris(hydroxymethyl)methylamine (Fisher Bioreagents, UK)
- TRITON X-100 (t-Octylphenoxypolyethoxyethanol) (Sigma, UK)

2.6.8 In-house Supplied Reagents

Reagent	Composition	Amount/litre
L-Broth/Agar	Bacto-tryptone	10.000g
	Yeast Extract	5.000g
	NaCl	10.000g
	Distilled Water	1.000l
Phosphate Buffered Saline	NaCl	10.000g
	KCl	0.250g
	Na_2HPO_4	1.437g
	KH_2PO_4	0.250g
	Distilled Water	1.000l
SOC medium	Deionised water	950.000ml
	Bacto-tryptone	20.000g
	Bacto yeast	5.000g
	NaCl	0.500g
	250mM KCl	10.000ml
	2M $MgCl_2$ solution	5.000ml
	1M sterile filtered glucose solution	20.000ml
Tris-acetate-EDTA (50x)	Tris Base	242.000g
	Glacial acetic acid	57.100ml
	EDTA	18.612g
	Distilled water	1.000l
Trypsin in Versene	NaCl	8.000g
	KCl	0.200g
	Na_2HPO_4	1.150g
	KH_2PO_4	0.200g
	EDTA	0.200g
	Trypsin	10.000g
	Phenol Red	0.015g
	Distilled Water	1.000l
Tris-borate EDTA (10x)	Trizma base	121.100g
	Boric acid	61.830g
	EDTA	18.600g
	Distilled water	1.000l

Table 2.7: In-house Supplied Reagents

Detailed preparation protocols can be found in (Sambrook and Russell, 2001).

2.6.9 Oligonucleotides

All oligonucleotides were synthesised by Eurogentec Oswel (0.04μM scale) and supplied in distilled water (See Tables 2.1 and 2.2).

2.6.10 Antibodies

ARP 3030 and ARP 3032, mouse derived IgG1-isotype monoclonal antibodies recognising HIV-2_{ROD} gp105, were obtained from the National Institute for Biological Standards and Controls, Centralised Facility for AIDS Reagents (NIBSC,CFAR) and were supplied as 50μl (65μg, 225μg respectively) purified antibody (Sattentau et al., 1993).

ARP 3083, ARP 3084, ARP 3085, ARP 3086, ARP 3087, ARP 3088, ARP 3089, ARP 3090, ARP 3091 (NIBSC, CFAR, UK) rat derived IgG2a/b-isotype monoclonal antibodies recognising HIV-2_{ROD} gp105 (for epitopes see Table 2.4) were supplied as 1ml culture fluid (McKnight et al., 1996).

NIH 1410 (NIH) rabbit derived polyclonal antiserum raised against HIV-2_{ST} gp105 was supplied as 200μl of antiserum (Ivey-Hoyle et al., 1991; Mulligan et al., 1992a).

Ab13412 (Abcam) is a mouse derived IgG1-isotype monoclonal antibody raised against the peptide AIEKYLKDQAQLNAWGCAFRQVC in HIV-2 gp36 and supplied as 50μg of a 1mg/ml purified antibody.

ARP 3002 and ARP 3044 (NIBSC, CFAR, UK) are mouse-derived IgG1-isotype monoclonal antibodies recognising SIV gp41 (mapped epitopes are aa595-617 and aa601-620 respectively) and were supplied as 100μl ascites and 1ml culture supernatant respectively (Kent et al., 1991; Kent et al., 1992).

Goat anti-mouse IgG (H+L) Horse Radish peroxidase conjugate (Promega, Cat. No. W402B 1mg/ml) was used at a dilution of 1:5000 to detect various primary antibodies in western blotting experiments.

Goat anti-rat IgG peroxidase conjugate (whole molecule) (SIGMA, Cat. No. A 9037) was used at a dilution of 1:1000 to detect ARP 3083 – ARP 3091 in western blotting experiments.

Goat anti-rabbit IgG peroxidase conjugate (whole molecule) (SIGMA, Cat. No. A-6154) was diluted 1:5000 to detect NIH 1410 in western blotting experiments.

2.6.10.1 Anti-His tag Antibodies

Supplier	Catalogue Number	Dilution for western blot
Sigma	A0758-IVL	1/2000
Qiagen	34660	1/2000
Novagen	70796-4	1/10,000

Table 2.8: Anti-His tag Antibodies

2.6.11 Bacteria

2.6.11.1 Bacterial Strains

- DH5α *Escherichia coli* library efficiency competent cells (Invitrogen Life Sciences, UK).
- Stbl2^{TM} Competent cells (Invitrogen Life Sciences, UK)
- SCS110 Escherichia coli competent cells (Stratagene)

2.6.11.2 Bacterial Media Supplements

- Ampicillin 50mg/ml stock (Sigma-Aldrich, UK)
- Nafcillin 50mg/ml stock (Sigma-Aldrich, UK)

2.6.12 Mammalian Cells

2.6.12.1 Media and Supplements

- CD CHO Medium (Invitrogen Corporation, UK)
- CHO III(A) Medium (Invitrogen Corporation, UK)
- CHO GS Medium (Invitrogen Corporation, UK) - *discontinued*
- Dulbecco's Modified Eagle Medium (DMEM)(Invitrogen Life Technologies, UK)

- Foetal Calf Serum (FCS) (PAA Laboratories), screened by in-house large-scale laboratory department for mycoplasma. Subsequently heat-inactivated at 56°C/30min.
- Foetal Calf Serum Dialysed (HD-FCS) (PAA Laboratories), pre screened by PAA for mycoplasma. Subsequently heat-inactivated at 56°C/30min.
- HT Supplement 50x (Invitrogen Life Technologies, UK)
- Interleukin-6 (IL-6) (Roche)
- L-glutamine – 200mM (in-house)
- L-methionine sulfoximine (MSX) (Sigma, UK)
- Optimem® I (Invitrogen Life Technologies)
- Penicillin/Streptomycin (P/S) supplied as a 100x stock solution of 10000U/ml Penicillin and 10mg/ml Streptomycin (Sigma-Aldrich,UK)
- Pepstatin A (Sigma, UK)
- RPMI 1640 L-glutamine free (Invitrogen Life Technologies, UK)
- Sodium Butyrate 98% (Aldrich, UK)

2.6.12.2 Cell Lines

All cell lines were incubated at 37°C in a 5% CO_2 atmosphere incubator unless otherwise stated.

- 293T cells are human embryo kidney cells transformed with SV40 large T antigen. Supplied by E. Yamada of the Edward Jenner Institute for Vaccine Research, Compton, UK. Cells were maintained in DMEM supplement with 10% heat inactivated FCS and 1% P/S.
- CV-1 cells are African Green Monkey kidney cells. CV-1 cells were already held in-house. Cells were maintained in DMEM supplemented with 10% heat inactivated FCS.
- CHO K1 cells are Chinese Hamster Ovary cells. CHO K1 cells were already held in-house. Cells were maintained in CHO-GS supplemented with 10% HD-FCS, 1% P/S and increasing concentrations of MSX >200μM(while producing stable cell lines) or in CHO III(A)/CHO CD supplemented with 1% HD-FCS with 1% P/S, 2% HT Supplement and 200μM MSX (when producing protein for studies).

•Hybridomas expressing ARP 3030, ARP 3032 and ARP 3083-3091 were provided by CFAR, NIBSC, UK as either frozen vials or live small flasks of cells. These cells were maintained as stated in section 2.20.2.8.1.

2.6.13 Stains and Reagents

2.6.13.1 Coomassie Blue Stain

Coomassie blue	1.25g
Methanol:water (1:1 v/v)	450ml
Glacial acetic acid	50ml
Total	**500ml**

Filter through a whatman No. 1 filter to remove any particulate matter.

2.6.13.2 Simply Blue Stain

Acid-based Coomassie blue G-250 staining solution. Prepare 0.2% (W/V) Coomassie brilliant blue G-250 (Bio-Rad) in water. Add an equal volume of 2N H_2SO_4. Mix well and let stand for 3h minimum. Filter through Whatman no. 1 filter paper. Measure recovered volume and add 1/9 vol of 10N KOH. Finally, add 100% (w/v) trichloroacetic acid (TCA) to a final concentration of 12% (w/v). The final concentration of Coomassie blue is ~0.08% (w/v). Store up to several months but maintain solution below pH 1.0.

2.6.13.3 Bradford Reagent

For 100ml of 5 x stock:

50mg Coomassie Brilliant Blue G-250
25ml ethanol
50ml 85% (v/v) Orthophosphoric acid
25ml DDW

Make up in sterile 50ml falcon tubes, avoiding any contact with glassware. Store in the dark at room temperature. Before use dilute with DDW and filter through a 0.45µm syringe top filter unit.

2.6.14 Buffers

2.6.14.1 RIPA Lysis Buffer

50mM Tris HCl pH 7.5

150mM sodium chloride

1% Nonidet P-40

0.5% sodium deoxycholate

0.1% SDS

2.6.14.2 Sample Loading Buffer

50mM Tris pH 6.8

5% 2-Hydroxyetylmercaptan β-mercaptoethanol

2% SDS

0.1% bromophenol blue

10% glycerol

2.6.14.3 SDS PAGE Running Buffer

50mM Tris(hydroxymethyl)methylamine

384mM Glycine

0.1% SDS/litre

2.6.14.4 Semi-Dry Blotting Buffer

14.5g Tris

7.475g Glycine

4.625ml 20% SDS

500ml (20%) Methanol/2.5 litre

2.6.14.5 Nickel Column Buffers

2.6.14.5.1 Binding Buffer 8x

4M NaCl

40mM Imidazole

160mM Tris HCl

2.6.14.5.2 Wash Buffer 8x

4M NaCl

480mM Imidazole

160mM Tris HCl

2.6.14.5.3 Elution Buffer 4x

2M NaCl

4M Imidazole

80mM Tris HCl

2.6.14.5.4 Strip Buffer 4x

2M NaCl

400mM EDTA

80mM Tris HCl

All these buffers were adjusted to a pH of 7.9 and working solutions were 1x.

2.6.14.6 Cobalt Column Buffers

2.6.14.6.1 Binding Buffer

pH 8

100mM NaCl

50mM NaH_2PO_4

10mM Tris HCl

2.6.14.6.2 Wash Buffer

pH7

100mM NaCl

50mM NaH_2PO_4

2.6.14.6.3 Elution Buffer

pH 6 / pH 5

100mM NaCl

50mM NaH_2PO_4

20mM PIPES

2.6.14.7 Concanavalin A Column Buffers

2.6.14.7.1 Binding Buffer

pH 7.4

0.5M NaCl

20mM Tris HCl

2.6.14.7.2 Elution Buffer

pH 7.4

0.5M NaCl

1M Methyl α D Glycopyranoside

20mM Tris HCl

2.6.14.7.3 Storage Buffer

pH6

1M NaCl

1mM $CaCl_2$

1mM $MnCl_2$

1mM $MgCl_2$

0.1M Acetate Buffer

2.6.14.8 GNA/PSA/HHL Column Buffers

2.6.14.8.1 Binding Buffer

Phosphate Buffered Saline

2.6.14.8.2 Wash Buffer

0.04M Methyl α-D-mannopyranoside in Phosphate Buffered Saline

2.6.14.8.3 Elution Buffer

0.5M Methyl α-D-mannopyranoside in Phosphate Buffered Saline

2.6.14.9 Ion Exchange Chromatography Buffers

2.6.14.9.1 Cation Exchanger Binding Buffers

pH 4 50mM Citric Acid

pH 5 50mM Acetic Acid

pH 6 50mM MES

pH 7 50mM Phosphate

pH 8 50mM HEPES

2.6.14.9.2 Anion Exchanger Binding Buffers

pH 5 20mM Piperazine

pH 6 20mM Piperazine

pH 7 20mM bis Trispropane

pH 8 20mM Tris

pH 9 20mM 1,3 diaminopropane

2.6.14.9.3 Elution Buffer

Add 1M NaCl to each of the above buffers.

2.6.14.10 Size Exclusion Chromatography

2.6.14.10.1 Superdex 200

pH 8

300mM NaCl

50mM Tris

2.6.14.10.2 Superose 6

pH 6.5

1M NaCl

50mM Hepes

Filter through 0.45µm membrane and degas before use.

2.6.14.11 Protein G Column Buffers

2.6.14.11.1 Start and Wash Buffer

pH 7

20mM Sodium Phosphate

2.6.14.11.2 Elution Buffer

pH 2.7

0.1M Glycine

2.6.14.12 Cyanogen Bromide Buffers

2.6.14.12.1 Coupling Buffer

pH 8.3

0.5M NaCl

0.1M $NaHCO_3$

2.6.14.12.2 Resin Buffer

1mM HCl

2.6.14.12.3 Blocking Buffer

pH 8

0.1M Tris HCl

2.6.14.12.4 Wash Buffers

pH 4

0.5M NaCl

0.1M Acetate Buffer

+

pH 8

0.5M NaCl

0.1M Tris HCl Buffer

2.6.14.12.5 Storage Buffer

pH 8

150mM NaCl

0.02% Azide

50mM Tris HCl

2.6.14.13 Immunoaffinity Buffers

2.6.14.13.1 High pH Conditions

2.6.14.13.1.1 Binding Buffer

pH 8

50mM Tris

2.6.14.13.1.2 Wash Buffer

pH 8

10mM Phosphate

2.6.14.13.1.3 Elution Buffer

pH 11.5

100mM triethylamine

2.6.14.13.1.4 Collection Buffer

pH 6.8

1M phosphate

2.6.14.13.2 Low pH Conditions

2.6.14.13.2.1 Binding Buffer

pH 8

50mM Tris

2.6.14.13.2.2 Wash Buffer

pH 5.5

10mM NaCl

50mM phosphate

2.6.14.13.2.3 Elution Buffer

pH 2.5

10mM NaCl

50mM glycine

2.6.14.13.2.4 Collection Buffer

pH 9.0

1M Tris

2.6.14.13.3 High Salt Conditions

2.6.14.13.3.1 Binding Buffer

pH 7.2

10mM NaCl

50mM Tris

2.6.14.13.3.2 Wash Buffer

pH 7.2

10mM Tris

2.6.14.13.3.3 Elution Buffer

pH 7.2

3.5M MgCl

10mM Tris

2.6.14.13.3.4 Collection Buffer

None

2.6.14.13.3.5 Storage Buffer for Immunoaffinity Column

pH 7.4

150mM NaCl

0.02% Azide

50mM Tris

2.6.14.14 ELISA Buffers

2.6.14.14.1 Sensitising Buffer

0.621g sodium hydrogen carbonate

0.275g sodium carbonate

made up to 100ml with DDW (pH 9.5)

2.6.14.14.2 Diluting Buffer

0.5g Marvel

50µl Tween 20

made to 100ml with PBS

2.6.14.14.3 Blocking Buffer

1.0g Marvel

Made to 100ml with PBS

2.6.14.14.4 Washing Buffer

19g sodium chloride

1ml Tween 20

made up to 2L with DDW

2.6.14.14.5 OPD Tablets (Sigma, Cat. No. P-9187)

Dissolve Urea (Gold) tablet in DDW (20ml) an hour before use.

Add hydrogen peroxide (silver) tablet just before adding to the plate.

Chapter 3

Results

3 Results

3.1 Construct Generation and Screening for HIV-2 gp120 expression

3.1.1 Patient Samples

3.1.1.1 Aim

The first step was to produce a range of clones (see section 2.1) for analysis based on primary isolate sequences derived from the samples collected in Caio, Guinea Bissau. This was to generate products from HIV-2 strains that might be more relevant to disease in the human population than the prototype, HIV-2_{ROD}, that has been cultured extensively. Six of the sixty-six expression competent *env*-genes rescued, were chosen to produce the clones based on their conserved cysteine and proline content. The various constructs involved mutations to the cleavage site between the globular head and transmembrane ectodomain of HIV-2 *env*-gene as well as the addition of the C-terminal domain of Fibritin (Yang et al., 2002) and a His-tag. The cleavage site and Fibrtin modifications were designed to aid the production of stable, soluble, trimeric envelope glycoproteins, whilst, the His-tag was added to aid purification of the expressed glycoprotein.

3.1.1.2 Amplification

In the following presentation, individual patient samples are named 1→6, and particular gp120 constructs (Figure 2.1) given the annotation (1,2,N,F) as presented in Table 3.1.

Construct	Construct Designation	Patient Designation					
		B1255 c1	**B1187 c9**	**B1183 c4**	**B1172 c4**	**B1286 c12**	**B1074 c10**
Patient No.		**1**	**2**	**3**	**4**	**5**	**6**
gp120	**1**	13	2	2	2	15	2
gp120FIB	**2**	3	5	2	2	N/T	2
gp120 cs	**N**	2	2	2	2	3	1
gp120FIB cs	**F**	4	1	N/T	1	1	2

Key

gp120 – HIV-2 *env*-gene encoding the ectodomain of gp140

FIB – fibritin trimerisation domain

cs – cleavage site mutant

Table 3.1: The number of *env*-gene containing colonies recovered

The numbers are shown for each construct, N/T indicates that no positive (*env*-gene containing) colonies were obtained following transformation of DH5α *E. coli*.

The HIV-2 *env*-gene fragments required to produce the designed gp120 products were generated by PCR off the pQ7 – *env* templates with the required primer pairs (Table 2.1b). Conditions relating to hybridisation temperature, elongation time and primer/template ratios were optimised for each PCR. First round PCR's all yield products of the expected sizes but for all patient samples, reactions employing primer H2140FIB yielded low amounts of product (Figure 3.1a). This is probably related to the large size of this primer (Table 2.1a). The cleavage site mutants were produced by second round PCR employing splice overlap extension (fragment C being joined to fragments D or E) creating amino acid substiutions at position 479 and 490 (K→E and R→T respectively) and again reactions employing H2140FIB were weak (Figure 3.1b).

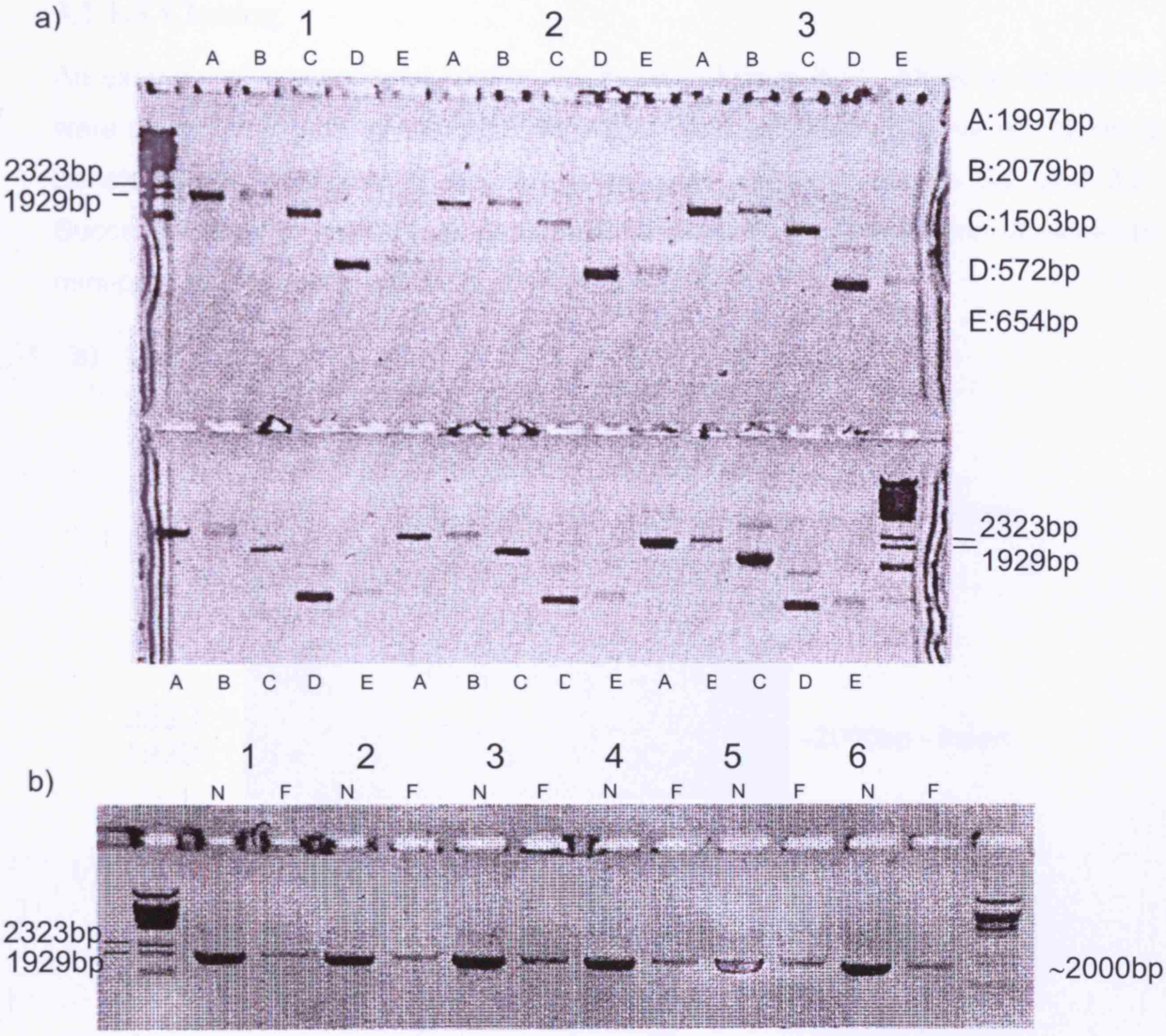


Figure 3.1: O.8% TBE/Agarose gel analysis of PCRs

a: First round PCR. Products analysed for individual patient samples (1→6). A: native *env*-gene (construct 1), B: *env*-gene with the fibritin coding domain on the 3' end (construct 2), C: the *env*-gene up to the processing site i.e. equivalent to gp105, D: spans the processing site to the 3' end, E: equates to D with the fibritin coding domain attached. Larger products were apparent for some products such as 3D, 6C and 6D, but, these were removed during purification of PCR products. **b:** Second round PCR yielding cleavage site mutated constructs. Products analysed for individual patient samples (1→6). The native *env*-genes with cleavage site mutations are construct N, and those with the fibritin coding domain are construct F.

3.1.1.3 Cloning

An example of a successful ligation is shown in Figure 3.2a. Whilst all constructs were successfully ligated into pEE14tPA2DCD4 only 22 were recovered following transformation of DH5α (Table 3.1). We were unable to clone 5.2 and 3.F. Successful transformation was confirmed by *Hind* III/*Eco* RI digestion of extracted mini-prep and midi-prep plasmid DNA (Figure 3.2b).

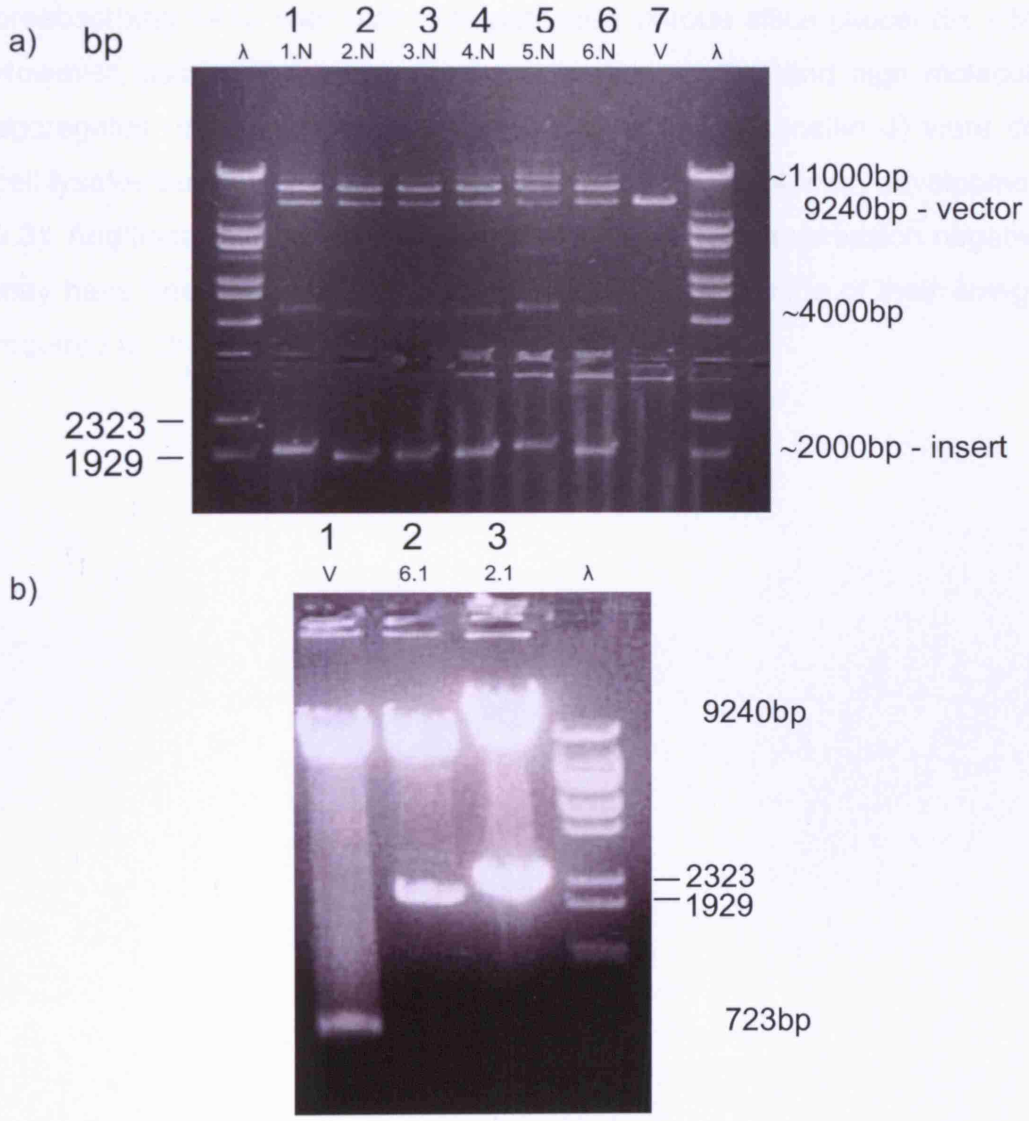


Figure 3.2: Examples of successful ligation and transformation

Samples were run on 0.8% TBE agarose gels. **a:** 10µl of a 20µl ligation mix were analysed for the samples indicated. Successful ligation is shown by the production of insert dimers and no products larger than the vector in the vector only lane (7). **b:** 5µl aliquots of the midi extracted plasmids shown here were digested with *Hind* III/*Eco* RI in 10µl reaction and the whole analysed. The release of a 723bp fragment from pEE14tPA2DCD4 (Lane 1) and the ~2Kb fragments of the *env*-coding genes (Lane 2 & 3) are clearly visible.

3.1.1.4 Transient Expression of Patient Samples

To screen for expression, transfections of 293T cells were set up from midi-prepared DNA using the Effectene protocol. Samples were collected at 72h post-transfection and stored for analysis. Due to cross reactivity of the polyclonal serum (NIH 1410 raised against HIV-2_{ST} gp105) in 293T cell lysates (results not shown), definitive identification of HIV-2 gp120 was difficult. This was overcome by preabsorbing 1410 with aldehyde-activated porous silica (Appendix - Method 1). However, using this preabsorbed preparation, gp120 and high molecular weight aggregates (despite gels being run under reducing conditions) were detected in cell lysates but not TCSN and the blots required long (45min) development (Figure 3.3). Additionally, a number of clones appeared to be expression negative but this may have been related to poor detection and sequencing of their *env*-genes was required to check if the reading frames were open.

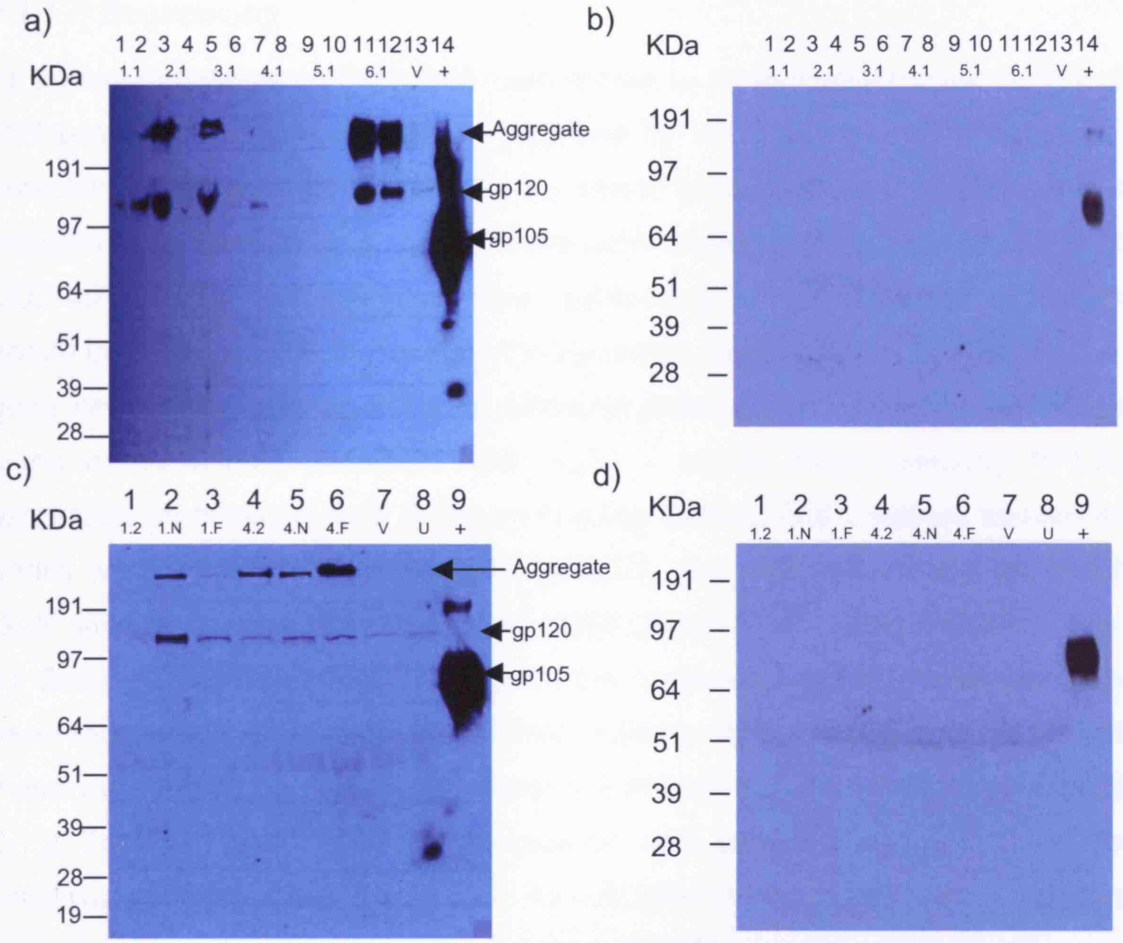


Figure 3.3: Western bolt detection of patient sample HIV-2 gp120 in cell lysates and TCSN

5-15% gradient PAGE gels were run, electro-blotted and probed with 1:1000 diluted preabsorbed serum 1410 (Appendix – Method 1) overnight at 4ºC. The secondary antibody was Goat anti-Rabbit at a dilution of 1:5000. The blots were exposed for 45min. Lanes 13 (**a** + **b**) and 7 (**c** + **d**) contain cell lysates and supernatant respectively from 293T cells transfected with pEE14tPA2DCD4. Lanes 14 (**a** + **b**) and 9 (**c** + **d**) contain the positive control EVA 621. **a:** Lanes 1→12 contain lysates for 2 clones each of the native gp120 (Construct 1) derived from all of the patients (1→6). **b:** Contain TCSN of the same samples as in (**a**). **c:** Lanes 1→3 and 4→6 relate to cell lysate samples containing patients 1 and 4 respectively for constructs 2, N and F as indicated, lane 8 contains non-transfected cell lysate. **d:** Contains TCSN of the same samples as in (**c**).

3.1.1.5 Sequencing

All patient-derived *env*-genes had been shown to be expression competent in the pQ7-based, T7-polymerase driven (supplied by VTF7-3) expression system with detection in western blot by anti-HIV-2_{ST} serum (see section 2.2.4) (Daniels et al., unpublished). Despite this, initial expression screens of *env*-constructs in the pEE14tPA2DCD4 system were poor, particularly so for detection of secreted gp120 in TCSN, based on the anti HIV-2_{ST} serum (see Figure 3.3). Therefore *env*-gene sequencing was undertaken following detection of expressed glycoprotein using a monoclonal antibody (ARP 3030 – tested when detecting HIV-2_{ROD} expressed products) to look for open reading frames. HIV-2 patient sample *env*-genes were sequenced using the MegaBACE and analysed using gap4 and the GDE suite of programmes (Flint et al., 1998; Smith et al., 1994) (Appendix Figure 4). Sequencing revealed that 17 expression competent patient sample clones had been recovered. These were from three patients, 2, 5 and 6 (Table 3.1). Mostly, these were based on construct 1 either the long or the short version (see section 3.1.2, Table 2.1 and Figure 3.5c), however, both patient 2 and 6 had one clone which was construct two (Table 3.1). As indicated in Appendix Figure 4 the clones sequenced correlated well with their respective pQ7-based parental clones.

3.1.1.6 Conclusion

This early section of the work proved difficult both during the cloning process and expression studies (discussed further in section 4.1). Finally, 22 out of a possible 24 constructs were produced and it is obvious from Table 3.1 that some clones were easier than others to obtain. Transient expression studies were attempted with these primary isolate clones but we were unable to detect soluble protein. As soluble proteins were the desired products the patient sample clones were temporarily set aside in favour of clones from HIV-2_{ROD} (See section 3.2). However, when detection of secreted product was evident with particular monoclonal antibodies for HIV-2_{ROD} (See Figure 3.6) I revisited the supernatants produced from 293T transient transfections with the primary isolate *env*-genes and identified 17 correctly sequenced expression competent clones fo further/future

study. Thus, this work was not in vain, it simply identified that further work was required before we could proceed.

3.1.2 HIV-2_{ROD}

3.1.2.1 Aim

The difficulties encountered with detection of HIV-2 gp120 derived from patient samples led us to try cloning and expression of the original laboratory isolate HIV-2_{ROD}. The majority of reagents available and/or obtained from CFAR and NIH were either raised against this isolate or recombinant proteins of it. Further, since the detection of patient derived HIV-2 gp120 in 293T cell TCSN was poor, it suggested that the glycoprotein was being retained either within the cell or on the cell surface. Examination of the C-terminus of the gp120 constructs (upstream of the proposed membrane anchor) revealed a stretch of 21 amino acids with a high hydrophobicity index that may have facilitated retention. Therefore new oligonucleotide primers were designed (Table 2.1) to allow removal of the *env*-gene sequence encoding these residues.

3.1.2.2 Cloning

With the new primers, a possible eight constructs could be generated (Table 3.2) for the HIV-2_{ROD} gp120. In addition construct 6 (short form) was generated for patients 5 and 6 off construct 1 (Appendix Figure 4). Out of these eight, seven were successfully ligated into pEE14tPA2DCD4 and transformed into *E. coli* DH5α.

Construct	Construct Designation	Number of expression competent *env*-genes
gp120 L	1	2 (28)
gp120FIB L	2	N/T (2)
gp120 cs L	34 (N)	1 (8)
gp120FIB cs L	35 (F)	1 (3)
gp120 S	6	3 (9)
gp120FIB S	7	2 (8)
gp120 cs S	38	3 (4)
gp120FIB cs S	39	2 (5)

Key

gp120 – HIV-2_{ROD} *env*-gene encoding the ectodomain of gp140

FIB – fibritin trimerisation domain

cs – cleavage site mutant

L – long form, containing the 21 amino acid hydrophobic region

S – short form, without the 21 amino acid hydrophobic region

Table 3.2: HIV-2_{ROD} *env*-genes cloned into pEE14tPA2DCD4

The number of expression competent clones of HIV-2_{ROD} *env*-genes recovered from DH5α *E. coli* cells are shown. Figures in parenthesis indicates the numbers of colonies obtained following transformation of DH5α *E. coli*. N/T indicates that stable transformants were not recovered for this construct.

Interestingly, whilst the gp120FIB L construct did yield bacterial colonies after 36-48h, such colonies did not grow in culture. Further, the colonies on the agar plates became flattened and non-viable after an additional 24h suggestive of bacterial lysis. The sequences of the gp120-Fibritin junction are shown (Figure 3.4). Although gp120 expression in bacteria has not been screened for, it appears that the bacterial lysis function is dependent on an intact gp105/gp36 processing site (as gp120FIB cs L was recovered successfully) and the 21 amino acid hydrophobic portion of gp36 (as gp120FIB S was recovered successfully).

Figure 3.4: Comparison of HIV-2_{ROD} gp120FIB constructs

Nucleotide and translation product sequences are shown for a: Fibritin upstream of the domain used in making the gp120FIB constructs (the N terminus of this domain is shown, GYIP), b: the long-form HIV-2_{ROD} constructs (only gp120FIB cs L cloned) and, c: the short-form HIV-2_{ROD} constructs (both gp120FIB S and gp120FIB cs S cloned). For the translation products amino acid properties are indicated: hydrophobic L, negatively charged D, positively charged K. At the nucleotide level tata boxes are highlighted.

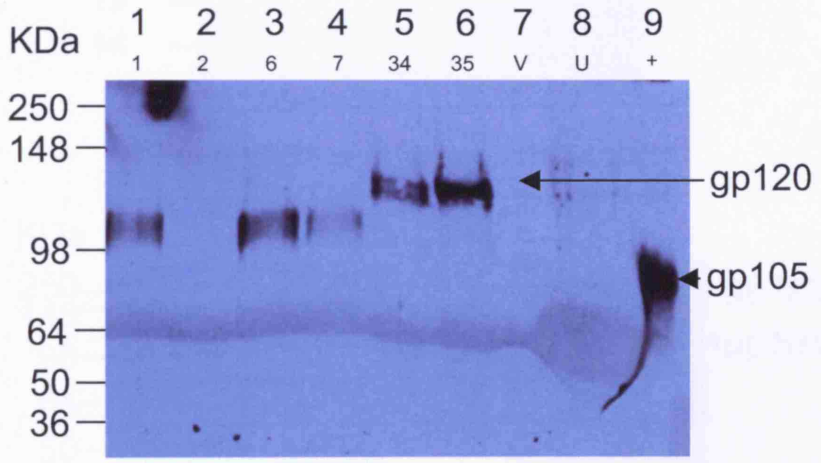
3.1.2.3 Transient Expression of HIV-2_{ROD}

Transfection of 293T cells for transient expression studies, monitored by western blot analysis (Figure 3.5a) and subsequent DNA sequencing identified 14 expression competent HIV-2_{ROD} *env*-gene clones that secreted products into TCSN (Table 3.2 and Figure 3.5b – not all shown). Given the detection success of probing TCSN with ARP 3032 and ARP 3030 (Figure 3.6), despite additional detection of serum proteins (possibly BSA), TCSN from 293T cells transfected with patient-derived gp120 clones were re-analysed and shown in some cases, probably dependent on sequence matching, to be positive (Figure 3.5c).

The failure to detect patient construct (gp120) with serum 1410 was surprising as this serum was used to detect all patient glycoproteins (full-length gp140) expressed using the pQ7-VTF7 vaccinia system in CV-1 cells (Daniels et al., unpublished). Lack of detection for the pEE14tPA/293T cell system presumably relates to subtle alterations in antibody reactivity between the two systems. In the pQ7 based system, the full-length native *env*-gene is present, retaining coding sequences for the natural signal peptide and cytoplasmic tail, expression is driven through a T7 promotor and CV-1 cells are inefficient at processing gp140 to gp105/36 (Moulard et al., 1999). In the pEE14-based system, the native signal

peptide is substituted with that of tPA (to improve yields of glycoprotein) and the membrane anchor and cytoplasmic tails are removed, expression is driven through a CMV promoter and 293T cells do process the encoded gp120 to gp105/gp15. The varied levels of expression in the pEE14-based system, possible differences in glycoprotein glycosylation between the CV-1 and 293T cell system and truncation to give gp120 constructs may all contribute to subtly alter the antigenicity of the extracellular domain (gp120) compared to gp140. In the latter context it has been reported that the removal of the cytoplasmic tail can alter the antigenicity of the SU component (Affranchino and Gonzalez, 2006; Edwards et al., 2002; Hoffman et al., 1999; Mulligan et al., 1992b; Spies et al., 1994)

a)



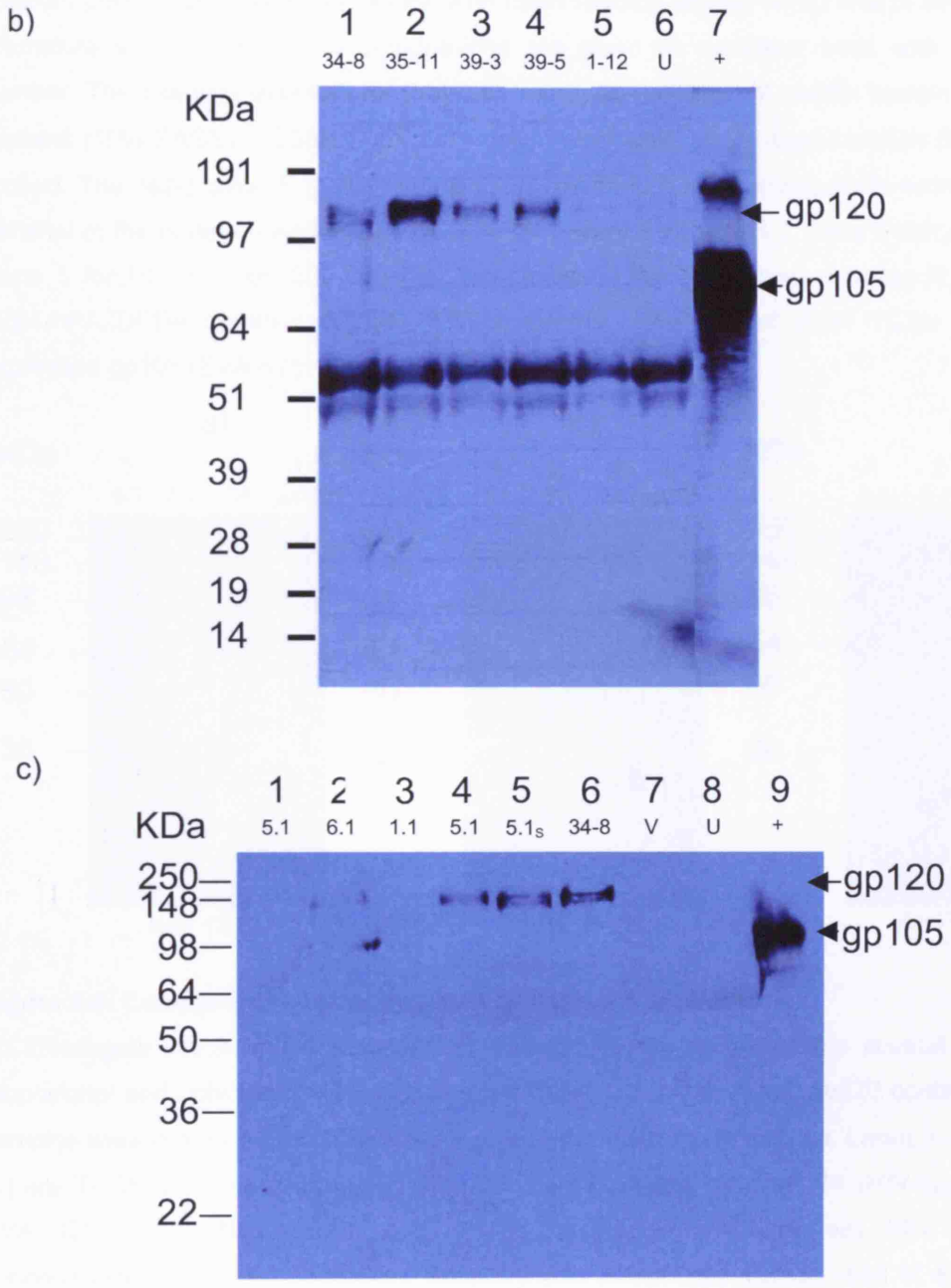


Figure 3.5: Screening for secreted HIV-2 gp120 following transient expression in 293T cells
Blots (**a**) and (**b**) used the primary monoclonal antibody ARP 3032 at a dilution of 1:2500 in 10% milk in PBT, whereas blot (**c**) was probed with ARP 3030 at a dilution of 1:2500. All three blots were incubated with the relevant primary antibody at room temperature for 90min, detected with Goat anti-Mouse IgG HRP incubated at room temperature for 60min at a dilution of 1:5000 in 10% milk in PBT (Table 2.3). **a:** Primary screen of HIV-2_{ROD} *env*-genes (10% PAGE). Lanes 1→4 were HIV-2_{ROD} gp120 constructs which contained premature stop codons (Appendix Figure 6). Lanes 5 + 6 were positive for HIV-2_{ROD} gp120 open reading frame (construct 34 and 35 respectively). The blot was exposed for 30s. **b:** Secondary screen of sequence confirmed HIV-2_{ROD} *env*-genes (5-15%

gradient gel), lanes 1→4 show clones with open reading frames whilst that in lane 5 contained a premature stop codon. Clone designations are given as construct code with appointed clone number. The blot was exposed for 2min. **c:** Improved detection of patient sample HIV-2 envelope proteins (10% PAGE). TCSNs from 293T cells transfected with patient samples (lanes 1→5) were probed. The designation 5.1_s indicates a short construct (i.e. 21 amino acids removed from the C-terminal of the protein – Appendix Figure 4) for patient 5 construct 1. Lane 6 contains construct 34 clone 8 for HIV-2_{ROD} gp120. The blot was exposed for 30s. Lanes labelled V, U, + contained pEE14tPA2DCD4 transfected 293T TCSN, control untransfected 293T TCSN and baculovirus expressed gp105 (EVA 621) respectively.

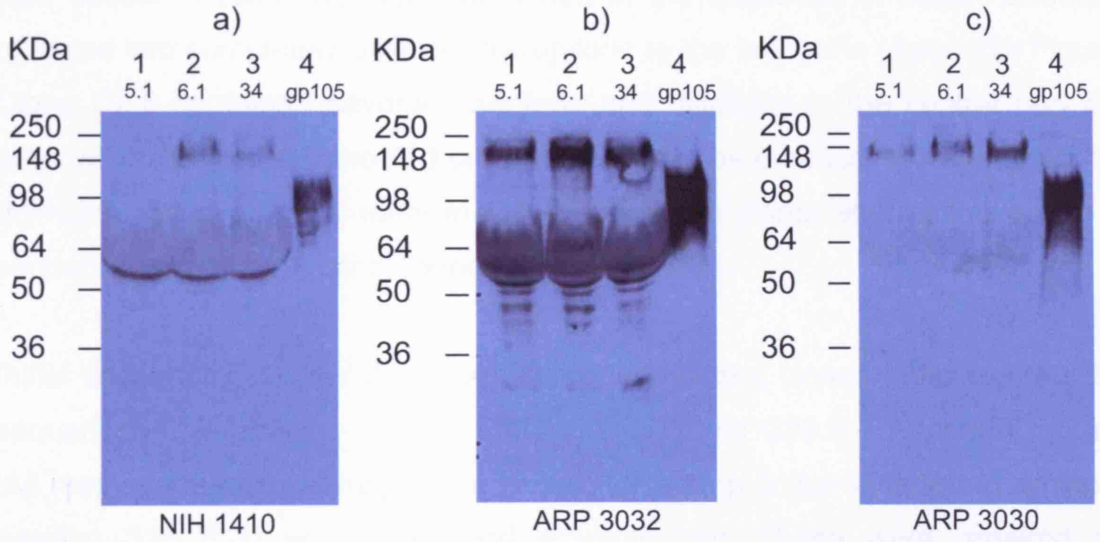


Figure 3.6: Comparison of effectiveness of different antibodies

To investigate which antibody to use in detection using western blots several antibodies, both monoclonal and polyclonal, were assessed against a panel of HIV-2 gp120 containing TCSNs. All samples were run on 5-15% SDS PAGE gels under reducing conditions. Lanes 1→3 in (**a**), (**b**) and (**c**) are TCSN expressing construct 5.1, 6.1 (HIV-2 patient samples) 34 (HIV-2_{ROD}) and lane 4 is EVA 621 (CFAR, NIBSC) HIV-2_{ROD} gp105. **a:** Primary antibody was NIH 1410 used at a concentration of 1:5000. Secondary antibody was a Goat anti-Rabbit used at a concentration of 1:5000. **b:** Primary antibody was ARP 3032 (CFAR, NIBSC) used at a concentration of 1:2000. **c:** Primary antibody was ARP 3030 (CFAR, NIBSC) used at a concentration of 1:500. Secondary antibody for both (**b**) and (**c**) was Goat anti-Mouse IgG HRP used at a concentration of 1:5000. All antibody incubations were at room temperature for 90min. All blots were exposed for 10s.

3.1.2.4 Sequencing

HIV-2_{ROD} gp120 *env*-genes were sequenced using the MegaBACE and analysed using gap4 and the GDE suite of programmes (Flint et al., 1998; Smith et al., 1994) (Appendix Figures 5 and 6).

The two clones recovered from HIV-2_{ROD} gp120 construct 2 either did not express or produced a truncated product. As this construct had been so difficult to clone (see section 3.1.2.1) we were interested in the sequence of these. Sequencing revealed two completely different disruptions to the *env*-gene (Appendix Figure 5). Clone C2.6 contained several insertions and deletions in the central part of the gene which caused a truncated protein product to be produced. Clone C2.27 had a thymidine base deletion early in C1 leading to a frame shift in the amino acid sequence and an early stop codon.

Initial sequencing of HIV-2_{ROD} expression competent clones indicated four intact sequences (designated - C34.8, C35.11, C39.3 and C39.5 – Appendix Figure 6), but many sequences were correct except for a stop codon inserted at amino acid position 318 (C3) which replaced a tryptophan. These were repaired using sequences C1.7, C6.26, C7.17, C38.39 and primers H2CORF (position 941-968 CCCAGACAAGCATGGTGCTGGTTCAAAG) and H2CORR (the reverse primer of H2CORF). This enabled the production of a further 10 clones (Appendix Figure 6) which were all shown to be expression competent (results not shown). Following this the sequencing of all 14 intact expression competent clones varied little from the HIV-2_{ROD} parental clone. However, in clone C39.5, the expressed product of which was used for all the analyses discussed later, there was an aspartic acid deletion within the Fibritin domain added to the C-terminal of the envelope glycoprotein. Despite this deletion the analyses shown later in this thesis indicated that the protein, designated 39.5 8, was both trimeric and correctly folded.

3.1.2.5 Stable Expression of HIV-2_{ROD} gp120

Given the more efficient detection of HIV-2_{ROD} gp120 products with the reagents available to us, experiments on constitutive expression of gp120 concentrated on the HIV-2_{ROD} *env*-gene clones. These were transfected into CHO K1 cells for the purpose of producing constitutively expressing cell lines. By approximately 2 months, post transfection, independent cloned populations of drug (MSX)-resistant cells were available for screening for production of HIV-2_{ROD} gp120 by western blot. Seven separate homogeneous populations of cells isolated by the individual clone selection method (see section 2.2.3.2.1), three for construct 1 and four for construct 39, produced HIV-2_{ROD} gp120 (Figure 3.7). These represented the native long form of gp120 (construct 1) and the short form fibritin domain containing cleavage site mutant (construct 39). As expected the former showed significant processing at the gp105/15 cleavage site resulting in a gp105 product whilst the latter was present as gp120 only (Figure 3.7).

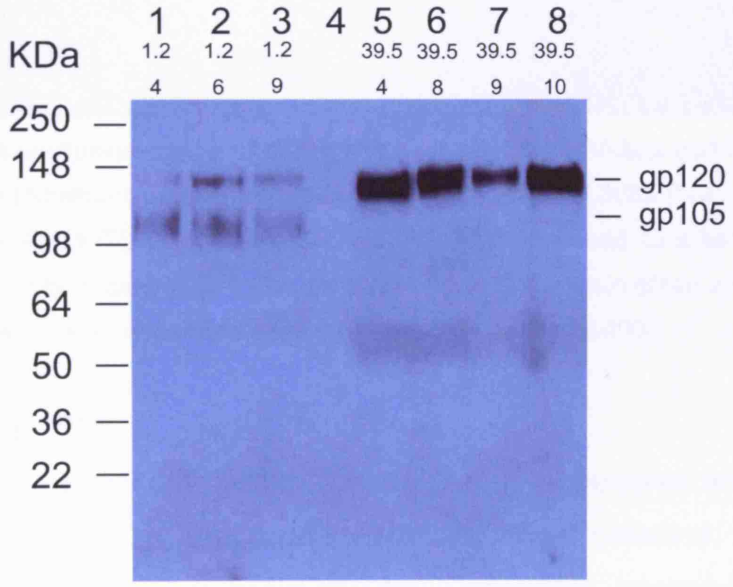


Figure 3.7: Western blot showing CHO K1 TCSN positive for HIV-2_{ROD} gp120 secretion

Lanes 1→3 and 5→8 are TCSN from CHO K1 cell lines expressing HIV-2_{ROD} gp120. Samples were run on 5-15% PAGE using reducing conditions. The primary antibody was ARP 3030 used at a dilution of 1:10 (Hybridoma TCSN) incubated at room temperature for 60min. The secondary antibody was an Goat anti-Mouse IgG HRP incubated at room temperature for 60min at a dilution of 1:5000 (Table 2.3). The blot was exposed for 1min.

3.1.2.6 Immunofluorescence

Immunofluorescent cell staining was used to determine what proportion of cells in the stable cell lines were expressing HIV-2_{ROD} gp120. Approximately 60% of the permeabilised cells were expressing HIV-2_{ROD} gp120 for cell line 39.5 8 and 30-40% for cell line 1.2 4. However, those cells which did express the glycoprotein gave strong immunofluorescence suggesting high level expression (Figure 3.8).

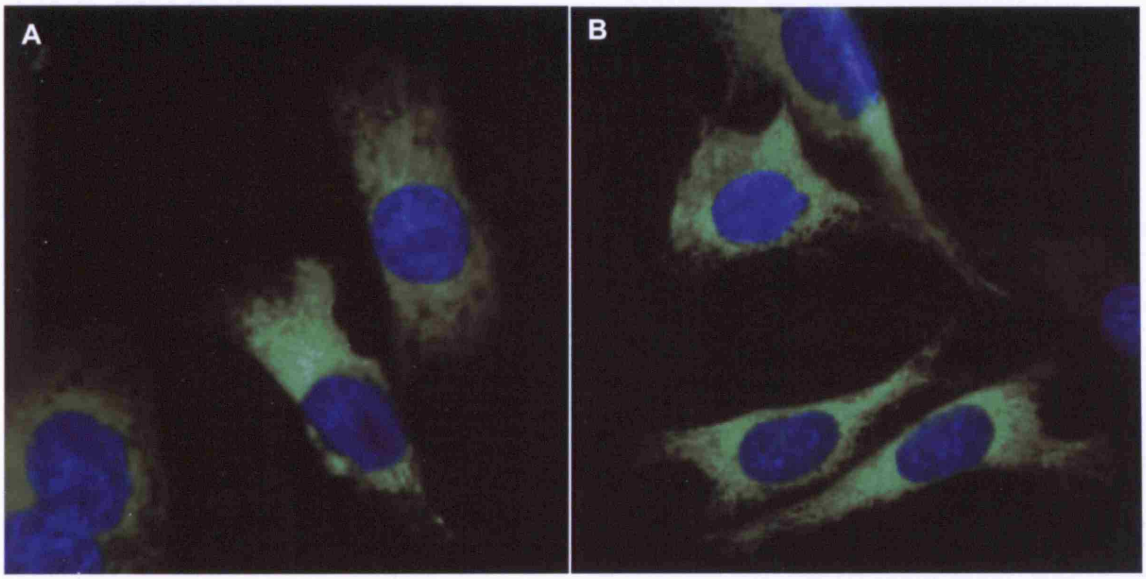


Figure 3.8: Immunofluorescence showing cells expressing HIV-2_{ROD} gp120 39.5 8
HIV-2_{ROD} gp120 (construct 39.5 8) was detected using: **A:** ARP 3085 (0.01mg/ml), **B:** ARP 3091 (0.01mg/ml). Anti-Rat FITC (Invitrogen, Cat. No. A21208) was used as a secondary antibody at a dilution of 1:100. In both cases the envelope glycoprotein is shown in green and the nucleus stained with DAPI is blue. These images are shown at a magnification of x 400.

3.1.2.7 Conclusion

This section of the work indicated that the use of monoclonal antibodies improved detection techniques so that secreted protein was detected. All constructs as shown in Figure 3.7 were secreted into the supernatant. However, I was not able to detect the C-terminal gp36 ectodomain with the reagents available to us, three specific gp36 antibodies (Table 2.3) and the anti-His tag antibodies (Section 2.6.10.1; results not shown). DNA sequencing of the expression competent *env*-clones indicated that we had 14 intact sequences for HIV-2_{ROD}. Following this constitutive cell line selection using methionine sulphoximine (MSX) was initiated. To achieve this a level of 200μM MSX was used to provide efficient killing on

non/poorly transfected cells, as determined from a CHO K1 kill curve (results not shown). This resulted in the production of seven stable cell lines of two different constructs (Construct 1 and 39, See Figure 3.7). Stable cell line production is a very time consuming process and this restricted repetition of the procedure. Therefore, the scale up of protein, optimisation of the purification protocol and functional and structural studies were determined based on constructs 1 and 39. It was evident from the immunofluorescence that further rounds of cell selection may have improved yield of protein. However, time restraints prevented further selection. The additional constructs could, at a later date, be used to produce stably transfected CHO K1 cell lines.

3.2 HIV-2_{ROD} gp120 Capture Systems

3.2.1 Aim

The aim of this part of the project was to produce HIV-2_{ROD} protein of a purity suitable for functional and structural studies, via the design and optimisation of a purification protocol.

3.2.2 Protein Production

Large volumes of TCSN were required to have sufficient quantities of protein for further experiments. Therefore, cells were grown to confluency in batches of twelve triple flasks at which time fresh medium (1.2l), incorporating 2mM Sodium Butyrate (Gorman, Howard, and Reeves, 1983) and 1µM of the acid protease inhibitor Pepstatin A (Sigma, Cat. No. P5318), was exchanged. Fresh Pepstatin A was added every day for three days to maintain the 1µM level, then on the fourth day the TCSN was harvested and Pepstatin A was added. The TCSN (1.2l) was clarified at 3,500rpm for 30min and concentrated using a 10K MWC hollow fiber filter to 120ml (concentrated 10 fold). The TCSN was then ready for purification.

3.2.3 HIS Columns

As all constructs contained a C-terminal hexa-His tag purification of these constructs should have been possible, at least partially, by utilising metal-ion affinity chromatography. However, the HIV-2 gp120 did not bind to either nickel or cobalt columns under a range of conditions, including when 8M Urea was added to all buffers (see section 2.3.4). The lack of availability of the hexa-His tags for binding was confirmed by the use of several commercially available anti-hexa-His tag antibodies (see section 2.6.14.5/6) which were unable to detect HIV-2 gp120 in western blots (results not shown).

3.2.4 Ion Exchange Chromatography

Attempts to purify HIV-2 gp120 on the basis of charge were made by using ion exchange chromatography. A pH series (see section 2.6.14.9) on both a cation (SP sepharose fast flow) and anion (Q sepharose fast flow) exchanger were used. pH values at the edge of the series, where the HIV-2 gp120 either bound to the column fully or had no binding affinity, indicated that the isoelectric point of HIV-2 gp120 was approximately pH 6. Following elution of bound material with elution buffer, relevant samples were analysed by silver staining. In all cases the HIV-2 gp120 was not being purified by this technique (Figure 3.9a).

In an attempt to improve the purity of the eluted HIV-2 Env protein, this protocol went through some optimisation. A wash step was added into the procedure using increasing molarities of sodium chloride to remove contaminants. This was attempted for pH 4 on the cation exchanger and pH 6 on the anion exchanger, as in these conditions HIV-2 Env bound to the exchanger (Figure 3.9a). However, the addition of increasing molarities of sodium chloride provided no observed difference in the purity of the eluted gp120 (Figure 3.9b).

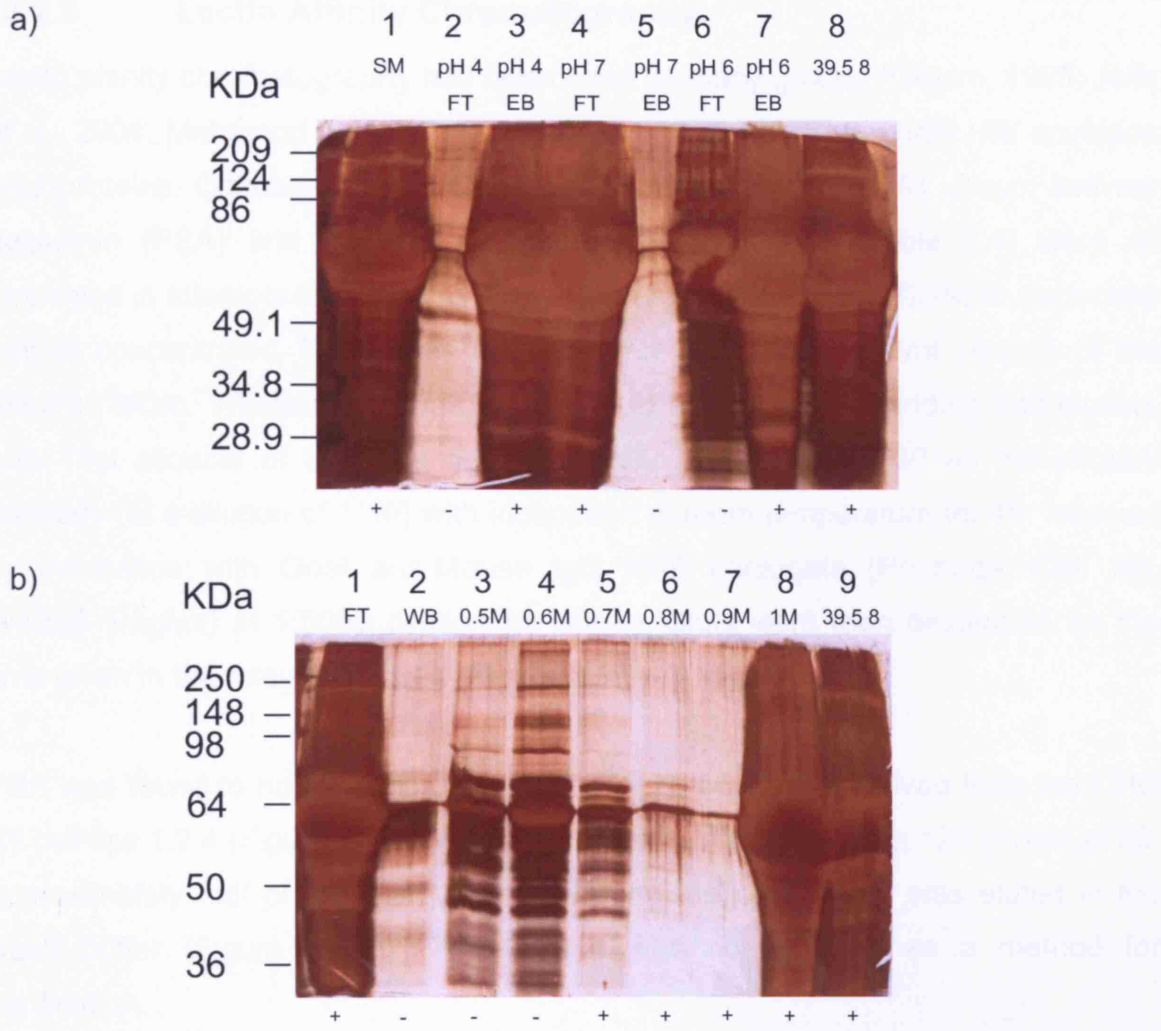


Figure 3.9: Silver Stain analyses of Ion exchange chromatography purification of HIV-2_{ROD} gp120

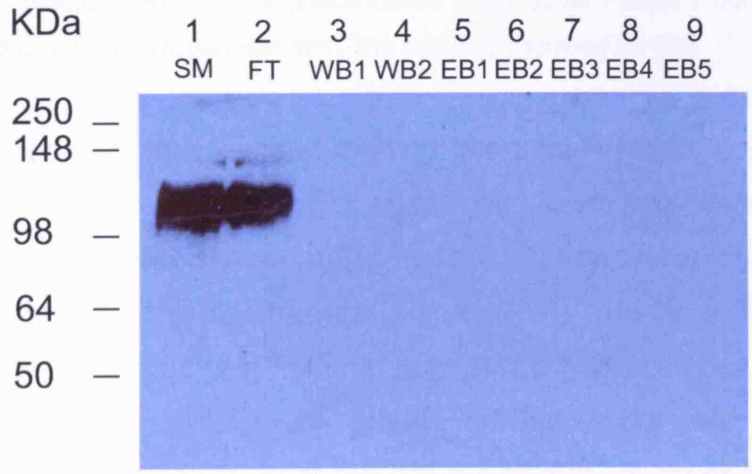
3ml of concentrated 39.5 8 TCSN (adjusted to the right pH) from CHO K1 cell line was the sample to be purified. All samples were run on 5-15% PAGE using reducing conditions. 15µl of each sample was run. **a:** Samples were those from the edge of the series on both the cation and anion exchangers. Lane 1 contained start material, lanes 2→5 were the flow throughs and elution buffers from pH 4 and 7 from the SP sepharose fast flow and lanes 6→7 were the flow through and elution buffer from pH 6 Q sepharose fast flow. **b:** Gel indicating that purity is not improved using increasing molarities of salt in the wash buffer on a SP sepharose fast flow run at pH 4. Lanes 1→2 were flow through and wash buffer respectively. Lanes 3→8 indicate the contaminants removed when washing the column with 0.5M to 1M NaCl. Lanes 5,6 and 7 were only weakly positive for HIV-2_{ROD} gp120. In the last lane of both gels 39.5 8 TCSN was run as an experimental control. Below the gels a summary of the results of western blot analysis for HIV-2_{ROD} gp120 is shown for positive (+) and negative (-) samples. For information on buffers used see section 2.6.14.9.

3.2.5 Lectin Affinity Chromatography

Lectin affinity chromatography has been used by many groups (Gilljam, 1993; Jeffs et al., 2004; Mahmood and Hay, 1992; Sourial et al., 2005) to purify HIV envelope glycoproteins. Concanavalin A (Con A), *Galanthus nivalis* (GNA), *Pisum sativum* agglutinin (PSA) and *Hippeastrum* hybrid (HHL) lectins (Table 2.4) were all employed in attempts to purify HIV-2 gp120 from concentrated TCSN. In each case 1ml of concentrated TCSN was passed three times over a 1ml column of the required lectin. Western blot was employed to assess gp120 binding and elution, with 15µl aliquots of fractions being analysed using ARP 3030 as the primary antibody (at a dilution of 1:10) with incubation at room temperature for 1h, followed by incubation with Goat anti-Mouse IgG HRP conjugate (Promega, Cat. No. W402B 1mg/ml) at 1:5000 dilution for 30min. Blots were then developed for the time given in the relevant figures (Figure 3.10 – 3.15).

PSA was found to have no binding affinity for HIV-2 gp120 derived from the CHO K1 cell line 1.2 4 (Figure 3.10). Whereas, when purifying HIV-2 gp120 construct 39, approximately half of the start material bound the column but was eluted in the wash buffer (Figure 3.10c). Therefore it was not pursued as a method for purification.

a)



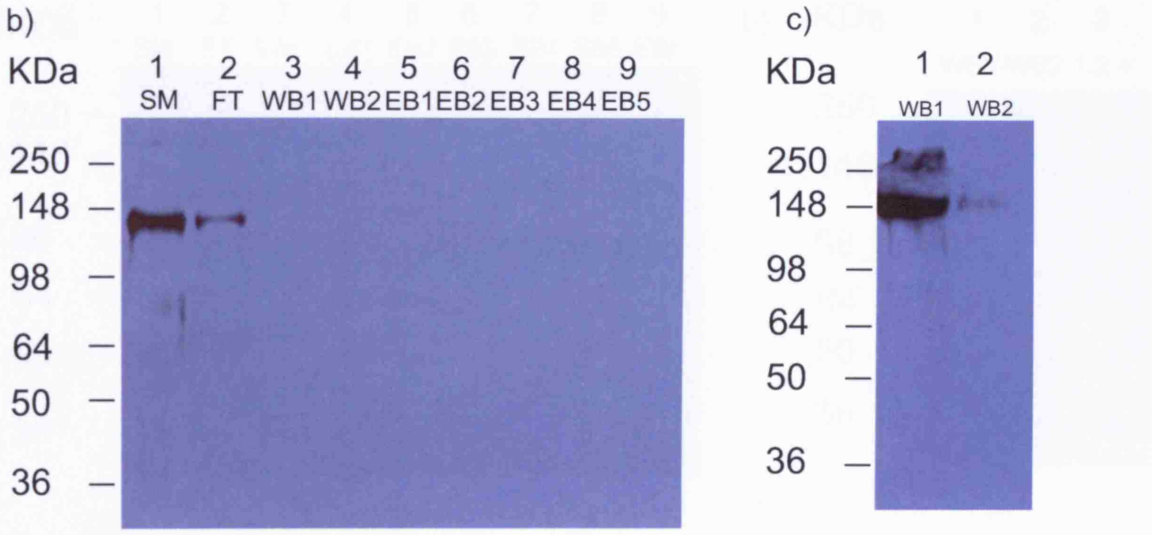


Figure 3.10: Western blot analyses of PSA Lectin Affinity Column purification of HIV-2_{ROD} gp120

Concentrated TCSN from 1.2 4 and 39.5 8 CHO K1 cell lines was subjected to purification. The column was then washed with 10ml PBS containing 0.04M Methyl α-D mannopyranoside collected in 2 x 5ml fractions and eluted with 0.8M Methyl α-D mannopyranoside in 7 x 1ml aliquots. All samples were run on 5-15% PAGE using reducing conditions. **a:** Western blot of fractions from the 1.2 4 column. Lanes 1→4 are start material flow through and wash buffers 1 and 2 respectively. Lanes 5→9 are the elution fractions 1→5. **b:** Western blot of fractions from the 39.5 8 column. The lane designation is the same as in a. Western blot of elutions 6→7 is omitted for both experiments as the blots were negative for HIV-2 gp120. The blots were exposed for 10min and 30s respectively. **c:** Western blot of 10 x concentrated wash buffer fractions from the 39.5 8 column, 15µl of each concentrated fraction was run. The blot was exposed for 30s.

Con A was found to have distinct binding characteristics for constructs 1 and 39 gp120s (Figures 3.11 and 3.12). For construct 1 there was weak affinity between the Con A lectin and the SU portion of the gp120, however a significant amount of protein was lost in the the flow through (Figure 3.11). The silver stain of the positive elution fractions indicated that minimal purification had occurred (Figure 3.11c). In contrast, it bound construct 39 gp120 efficiently but with high affinity as demonstrated by the need to heat the column to 50ºC in order to elute the bound protein (Figure 3.12).

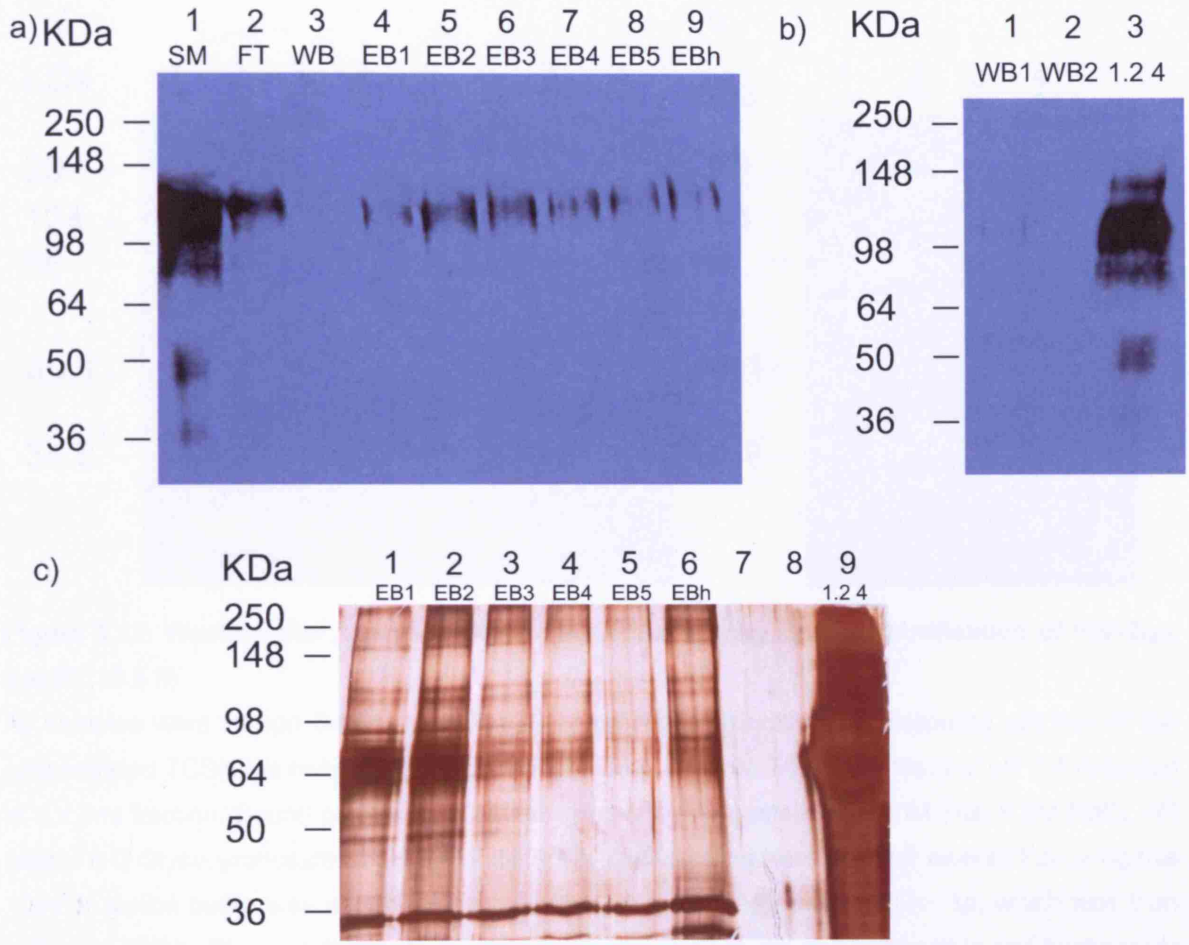


Figure 3.11: Western blot and Silver Stain analyses of Con A Lectin Affinity Column purification of HIV-2_{ROD} gp120 (1.2 4)

All samples were run on 5-15% SDS PAGE using reducing conditions. Column was run as described in Figure 3.11 except for the addition of 10ml of elution buffer at the incubation step. **a:** Lanes 1→3 are start material, flow through and wash buffer respectively, lanes 4→8 are the elution fractions 1→5, lane 9 contains the batch elution from the 1h at 50ºC. **b:** Western blot of fractions following 50ºC incubation, lanes 1→2 are the elution fractions 1→2 following the incubation step. In lane 3 is 1.2 4 TCSN run as a positive control. The blots were exposed for 30s. **c:** Silver stain of elutions 1→5 and the elution of the heat step. In lane 9, 1.2 4 was run as a positive control.

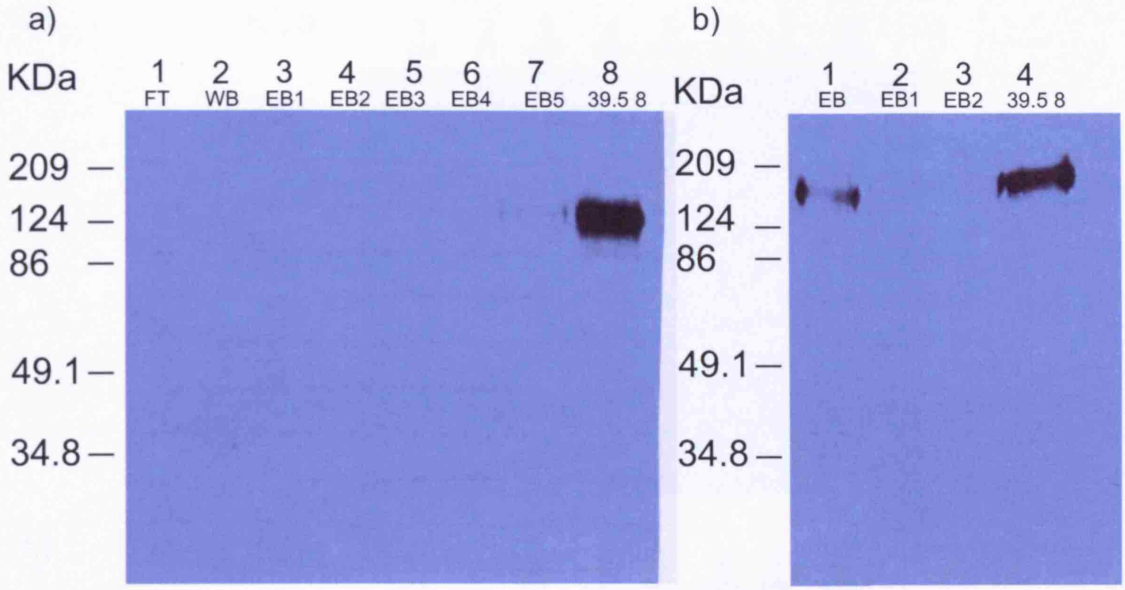


Figure 3.12: Western blot analyses of Con A Lectin Affinity Column purification of HIV-2_{ROD} gp120 (39.5 8)

All samples were run on 5-15% SDS PAGE using reducing conditions. Following addition of the concentrated TCSN the column was washed with 5ml of 20mM Tris, 0.5M NaCl at pH 7.4 collected in 1 x 5ml fraction. Bound protein was eluted using a buffer containing 20mM Tris, 0.5M NaCl, 1M Methyl α D Glycopyranoside at pH 7.4, initially 5 x 1ml fractions were used for elution. Following this 10ml of elution buffer was added to the column and it was heated at 50°C for 1h, which was then collected. Finally 2ml of elution buffer was passed over the column and collected in 1ml fractions. **a:** Western blot of fractions from the column. Lanes 1 + 2 contain samples of the flow through and the wash buffer respectively. Lanes 3→7 are elution fractions 1→5. **b:** Western blot of elutions following incubating the column at 50°C for 1hr. Lane 1 was the elution fraction after the heat step, lanes 2 + 3 are elution fractions 1 + 2 from rinsing the column following heating. In the last lanes of gels **a** + **b** 39.5 8 TCSN was run as a positive control. The blots were exposed for 2min.

HHL bound construct 39.5 8 efficiently and bound protein could be recovered in high yield by elution at room temperature (Figure 3.13b). In contrast, whilst HHL bound the unprocessed component of construct 1.2 4 efficiently, it gave low binding of the processed gp105 component (Figure 3.13a). However, both these bound components were eluted at room temperature.

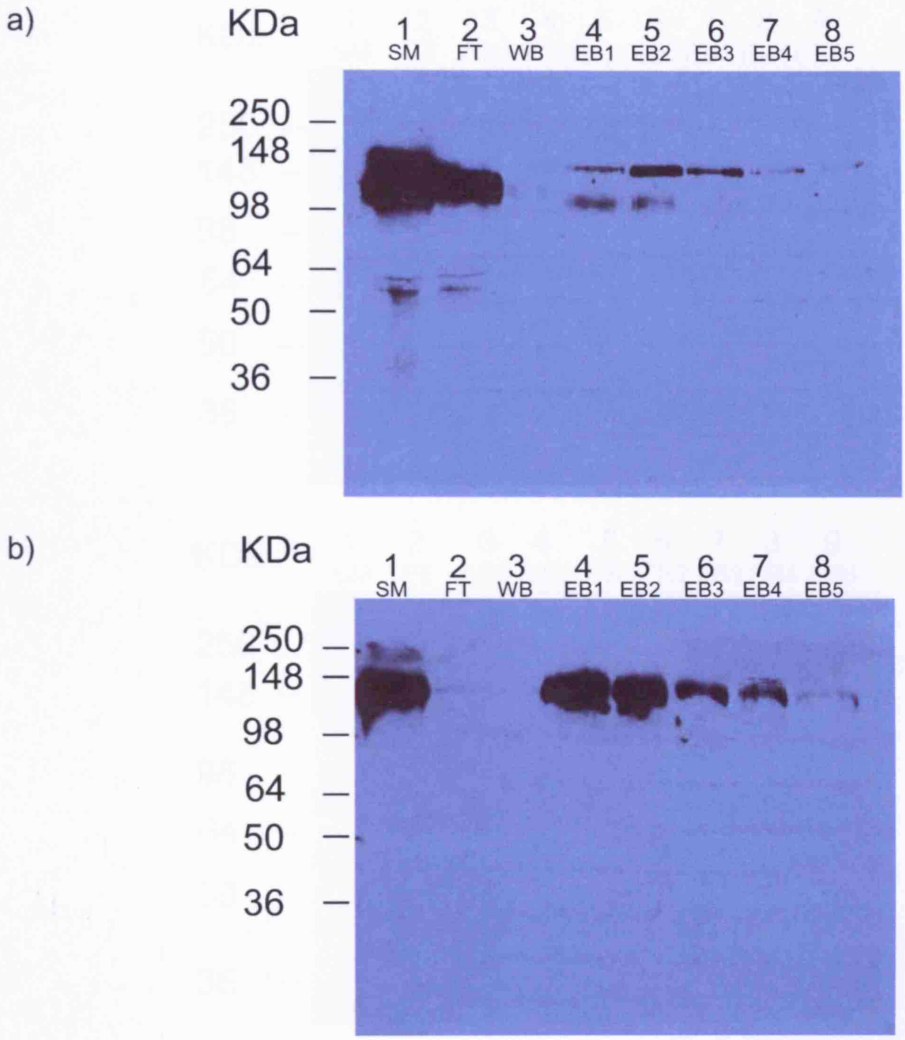


Figure 3.13: Western blot analyses of HHL Lectin Affinity Column purification of HIV-2_{ROD} gp120

Following addition of the relevant concentrated TCSN the column was washed with 25ml PBS containing 0.04M Methyl α-D mannopyranoside and eluted with 0.8M Methyl α-D mannopyranoside in 10 x 1ml aliquots. All samples were run on 5-15% PAGE using reducing conditions. **a:** Western blot of fractions from the 1.2 4 column. Lanes 1→3 are start material, flow through and wash buffer respectively. Lanes 4→8 are the elution fractions 1→5. **b:** Western blot of the fractions from the 39.5 8 column. The lane designation is the same as in a. Western blot of elutions 6→10 is omitted for both experiments as the blots were negative for HIV-2 gp120. The blots were exposed for 2min.

GNA bound construct 1 very poorly whereas this lectin had a high affinity for construct 39 (Figure 3.14). The latter HIV-2 gp120 was purified substantially, in comparison to HHL purified HIV-2 gp120 (39.5 8) as the band was distinguishable on a silver stained gel (Figure 3.15a and b).

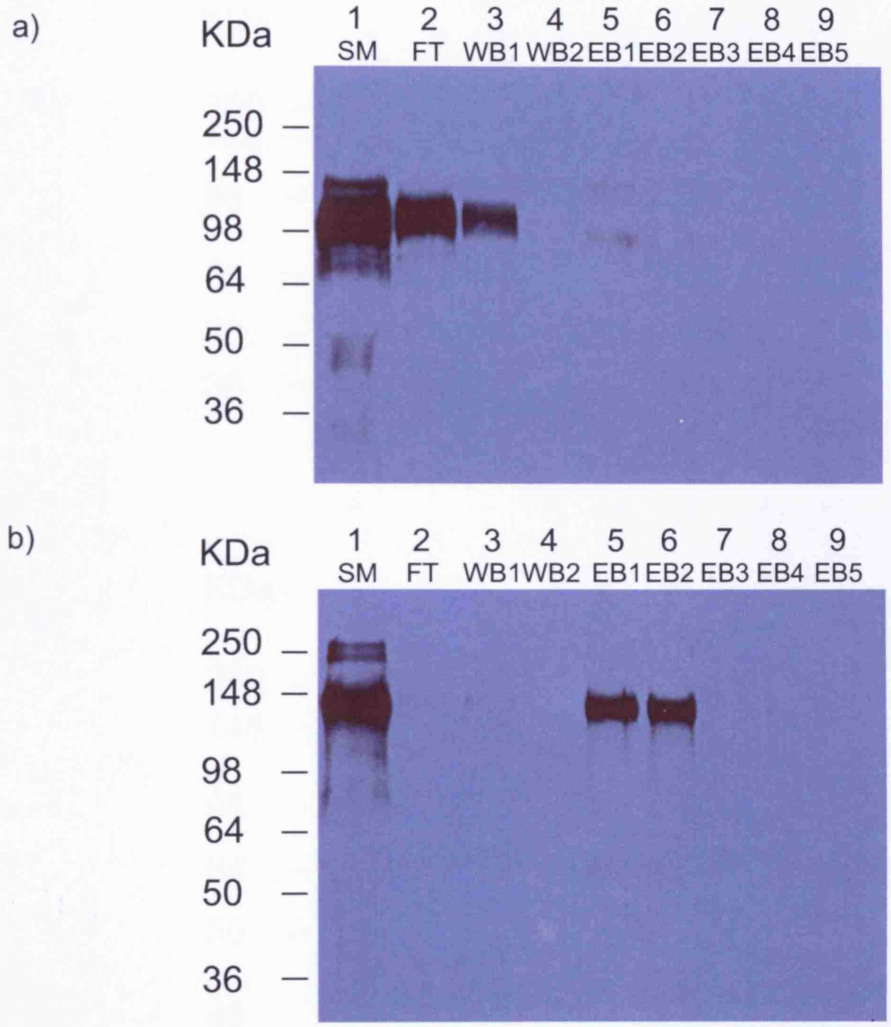


Figure 3.14: Western blot analysis of GNA Lectin Affinity Column purification of HIV-2_{ROD} gp120

Following addition of the relevant concentrated TCSN the column was then washed with 10ml PBS containing 0.04M Methyl α-D mannopyranoside collected in 2 x 5ml fractions and eluted with 0.8M Methyl α-D mannopyranoside in 7 x 1ml aliquots. All samples were run on 5-15% PAGE using reducing conditions. **a:** Western blot of fractions from the column when purifying 1.2 4. Lanes 1→4 are start material, flow through and wash buffers 1 and 2 respectively. Lanes 5→9 are the elution fractions 1→5. **b:** Western blot of fractions from the column when purifying 39.5 8, lane designation is the same as in a. Elution fractions 6→7 from both purifications were omitted as they were negative for HIV-2 gp120. The blots were exposed for 30s.

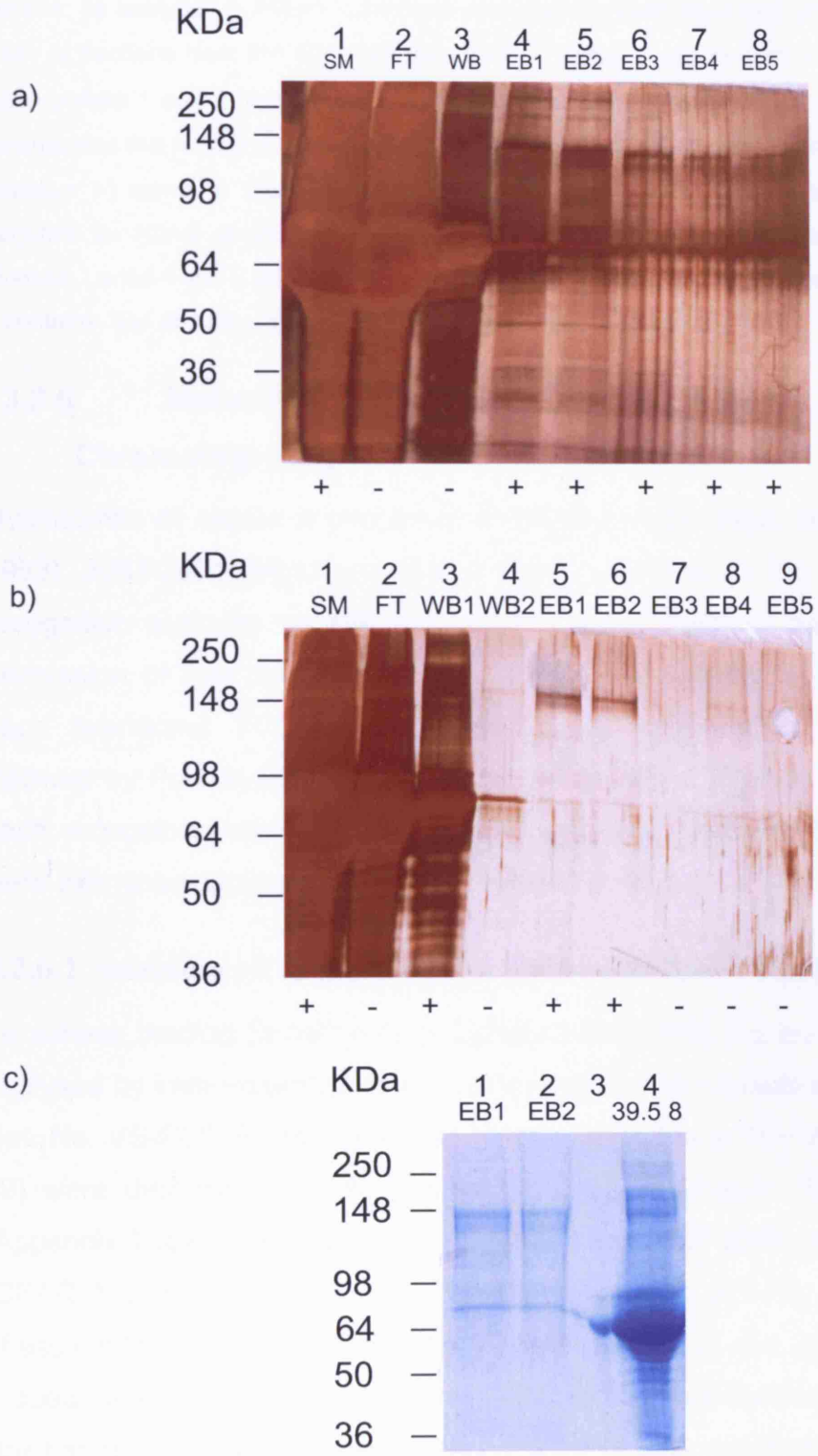


Figure 3.15: Silver Stain and Simply Blue Stain analyses of GNA and HHL Lectin Affinity Column purification of HIV-2_{ROD} gp120

All samples were run on 5-15% PAGE using reducing conditions. **a:** Fractions from HHL column, as analysed in Figure 3.13b were screened by silver stain. Lanes 1→3 are start material, flow through and wash buffer respectively. Lanes 4→8 are the elution fractions 1→5. **b + c:** Fractions from GNA

column, as analysed in Figure 3.14 were screened by silver stain and simply blue stain. **b:** Silver stain of fractions from the GNA purified 39.5 8. Lanes 1→4 are start material, flow through and wash buffers 1 and 2 respectively. Lanes 5→9 are the elution fractions 1→5. Below gels a and b summarises the results of western blot analysis for HIV-2_{ROD} gp120 are shown for positive (+) and negative (-) samples. Elution fractions 6→7 from both purifications were omitted as they were negative for HIV-2 gp120. **c:** Simply Blue Stain of 39.5 8 GNA purified HIV-2 gp120 positive elutions. Lanes 1 and 2 are elution fractions' with 2.7µg of total protein being run in each lane. Lane 4 contains 10µl of concentrated 39.5 8 TCSN as a positive control.

3.2.6 Monoclonal Antibody Based Immunoaffinity Chromatography

Hybridomas of eleven monoclonal antibodies, ARP 3030, 3032 (Sattentau et al., 1993), 3083-3091 (McKnight et al., 1996), selected on the basis of their known recognition epitopes on HIV-2_{ROD} gp105 were obtained from NIBSC, with kind permission of their contributors (see Table 2.4). Initially, approximately 250ml of each hybridoma TCSN was purified using ammonium sulphate precipitation followed by Protein G chromatography (see section 2.3.7.3), to supply enough of each monoclonal antibody to prepare individual immunoaffinity columns, which were examined against HIV-2 gp120 construct 39.5 8.

3.2.6.1 Selection of MAb to use in Immunoaffinity Chromatography

To assess binding to native (non-denatured) gp120, the purified antibodies were analysed by immunoprecipitation, native PAGE and vivaspin columns (Vivascience Cat. No. VS-PC01EPPC) and their binding affinities to HIV-2_{ROD} gp120 (construct 39) were determined using surface plasmon resonance (BIAcore). The results (Appendix Table 2 and Table 3.3) indicated that ARP 3085 (McKnight et al., 1996) (CFAR, NIBSC), due to its ability to interact strongly with HIV-2 gp120 in a number of assays (shown in Table 3.3 and Appendix Table 2) and 22nM affinity, would be a good candidate for an immunoaffinity column. This hybridoma was grown in 20 litre batches which were then concentrated to approximately 500ml by the large scale laboratory, NIMR. The hybridoma TCSN was purified as described previously (Section 2.3.7.3). The purified ARP 3085 was then coupled to sepharose using cyanogen bromide. In total over 200mg of ARP 3085 was coupled to sepharose to produce a 29ml column.

Antibody ARP[a]	Epitope[b]	Concentration mg/ml[c]	K_D[d]	Native Gel[e]	Immuno-precipitation[e]	Vivaspin Column[e]
3083	*44.5j* – C1 DDYQEITLNVTE	0.640	8.58×10^{-9}	++	++	++
3084	*25.8c* – V1 SEDTPCARA	0.549	1.47×10^{-8}	++	++	++
3085	*44.5k* – V2 GEEETINCQ	0.398	2.17×10^{-8}	++	+++	+++
3086	*44.2g* – V2 FNMTGL	0.835	1.91×10^{-8}	++	++	N/D
3087	*28.3e* - CD	0.622	2.77×10^{-7}	+	+++	+++
3088	*28.8e* - CD	0.346	7.25×10^{-8}	+++	+++	+
3089	*25.3f* - CD	0.568	2.57×10^{-6}	+	+++	+
3090	*32.2f* – V3 LMSGHVFHSHYQ	0.429	5.47×10^{-6}	+	–	–
3091	*32.7g* – V3 SGHVFHSHYQ	0.353	6.26×10^{-8}	+++	+	+
3030	*PQ41* - C2	2.033	8.14×10^{-7}	+	+	+++
3032	*PQ2G12* - C1	5.492	5.28×10^{-8}	+	+	+++

Table 3.3 Results of Immunoaffinity Experiments

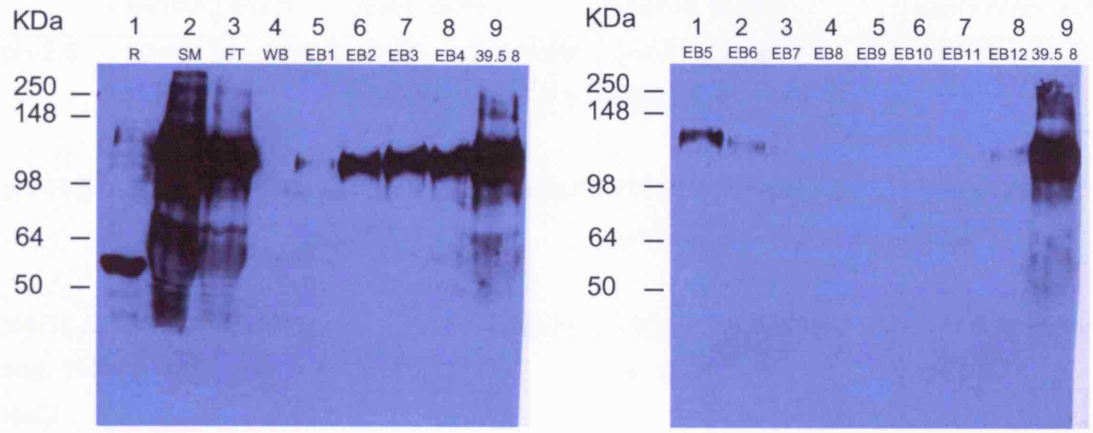
[a]The antibodies are described by their ARP No. assigned by CFAR (NIBSC, UK). [b]Italics shows the designation given to the antibody in the producers' original papers (McKnight et al., 1996; Sattentau et al., 1993). C = Constant domain, V = Hypervariable domain, CD = conformation dependant. Where known, the precise epitope determined by mapping with HIV-2_{ROD} gp105 peptides is shown. [c]Concentration of stock purified antibodies is shown. [d]K_D was determined using SPR (BIAcore) with HIV-2_{ROD} gp120 bound to the CM5 chip. [e]Gels were run for HIV-2_{ROD} gp120 as indicated (native/IP/Vivaspin) and blotted with the individual antibodies: +++ indicates a very strong response on western blot, ++ indicates a strong response , + indicates a weak response and – indicates no response (see Appendix Table 2). N/D – Not Done.

3.2.6.2 Determination of Conditions for Immunoaffinty Chromatography

Several conditions for immunoaffinity chromatography were assessed. Elution conditions such as acid pH (pH 2.5), alkaline pH (pH 11) (Figure 3.16) and high molarity salt (3.5M Magnesium Chloride) were trialled. The protocol which utilised high molarity salt produced very low yields of eluted protein, hence this was not further investigated (results not shown). Small columns were used for all of these trials and therefore overloading occurred shown by the presence of HIV-2_{ROD} gp120 in the flow throughs. Figure 3.16a (elution at pH 2.5) indicates a sharper

elution profile than elution at pH 11.5 (Figure 3.16b). However, the latter could be improved by adding 1M NaCl to the wash buffer (Figure 3.16c). Obviously, western blots give no indication of the purity of the eluted fractions, hence these fractions were subjected to silver staining (Figure 3.17). The results of the silver stain showed that although eluting at pH 11.5 provided the greatest yield of HIV-2_{ROD} gp120 no purification of the sample occurred (Figure 3.17b), although the purity was improved by adding 1M NaCl to the wash buffer (Figure 3.17c). However, purity was similar to the latter result but yields appeared greater when a standard pH 8 binding, pH 6.8 wash and a pH 2.5 elution was employed (Figure 3.17a). Therefore, this method was used in the purification protocol.

a)


KDa
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R SM FT WB EB1 EB2 EB3 EB4 39.5 8
250
148
98
64
50
KDa
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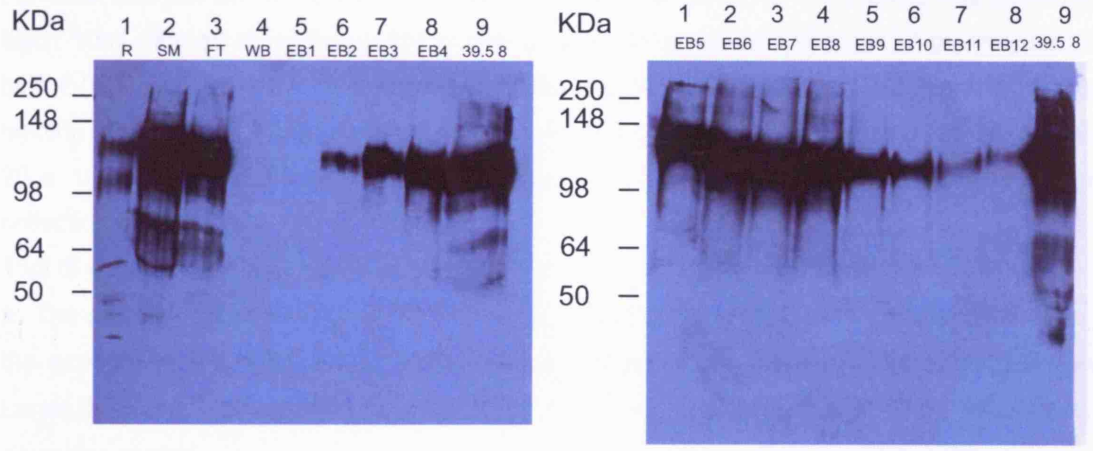
EB5 EB6 EB7 EB8 EB9 EB10 EB11 EB12 39.5 8
250
148
98
64
50

b)


KDa
1 2 3 4 5 6 7 8 9
R SM FT WB EB1 EB2 EB3 EB4 39.5 8
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KDa
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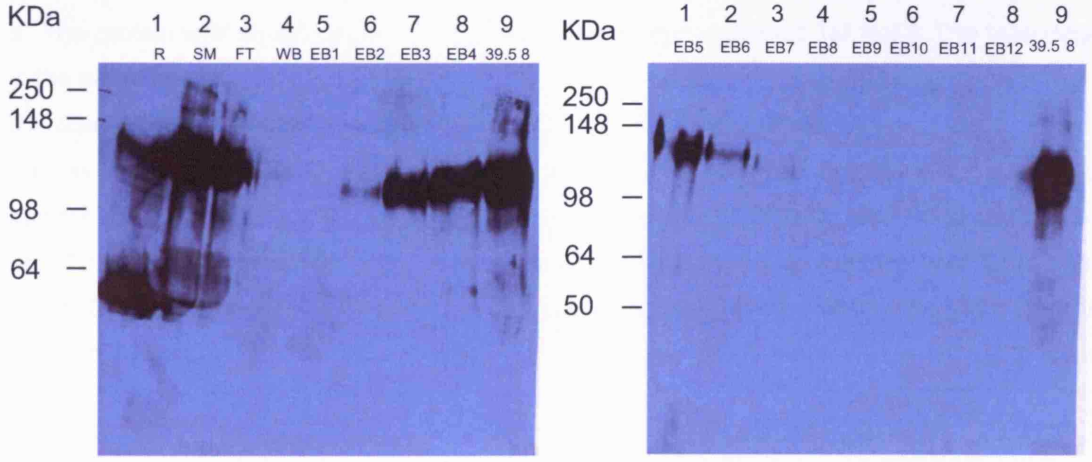

KDa
1 2 3 4 5 6 7 8 9
R SM FT WB EB1 EB2 EB3 EB4 39.5 8
250
148
98
64
KDa
1 2 3 4 5 6 7 8 9
EB5 EB6 EB7 EB8 EB9 EB10 EB11 EB12 39.5 8
250
148
98
64
50

d)

	Binding Buffer	Wash Buffer	Elution Buffer	Collection Buffer
pH 2.5	50mM Tris pH 8	50mM phosphate 10mM NaCl pH 6.8	50mM glycine 10mM NaCl pH 2.5	1M Tris pH 9.0
pH 11.5	50mM Tris pH 8	10mM Phosphate pH 8	100mM triethylamine pH 11.5	1M phosphate pH 6.8
pH 11.5 and 1M NaCl	50mM Tris pH 8	10mM Phosphate, 1M NaCl pH 8	100mM triethylamine pH 11.5	1M phosphate pH 6.8

Figure 3.16: Western blot analyses of immunoaffinity column purification of HIV-2_{ROD} gp120

For each analysis 2ml of concentrated 39.5 8 TCSN from the CHO K1 cell line was diluted to 10ml. Each 10ml (filtered through a 0.45µm membrane and adjusted to the pH of the required binding buffer (**d**)) was passed over the column following equilibration of the column with the relevant binding buffer. The column was then washed with 20ml of the relevant wash buffer (**d**) and eluted in 20 x 1ml aliquots of elution buffer (**d**). The pH was adjusted to a neutral pH by the relevant collection buffer (**d**).

15µl of each sample was run on a 5-15% PAGE using reducing conditions.

a: The protein was eluted at pH 2.5. Lanes 1→4 are resin from the immunoaffinity column before the experiment (R), 39.5 8 start material, flow through (FT) and wash buffer (WB) respectively. Lanes 5→8 and 1→8 are the elution fractions 1→12. In lane 9 of both gels 39.5 8 TCSN was run as a positive control.

b: The protein was eluted at pH 11.5. The lane designation is the same as in a.

c: The protein was eluted at pH 11.5 but the wash buffer contained 1M NaCl. The lane designation is the same as in **a**.

d: Table indicating buffers used in each experiment mentioned above.

Elution fractions 13→20 of each experiment were not included as they were negative by western blot for HIV-2_{ROD} gp120. The primary antibody in western blotting was ARP 3030 used at a dilution of 1:10 incubated at room temperature for 60min. The secondary antibody was Goat anti-Mouse IgG HRP incubated at room temperature for 30min at a dilution of 1:5000. The blots were exposed for 2min.

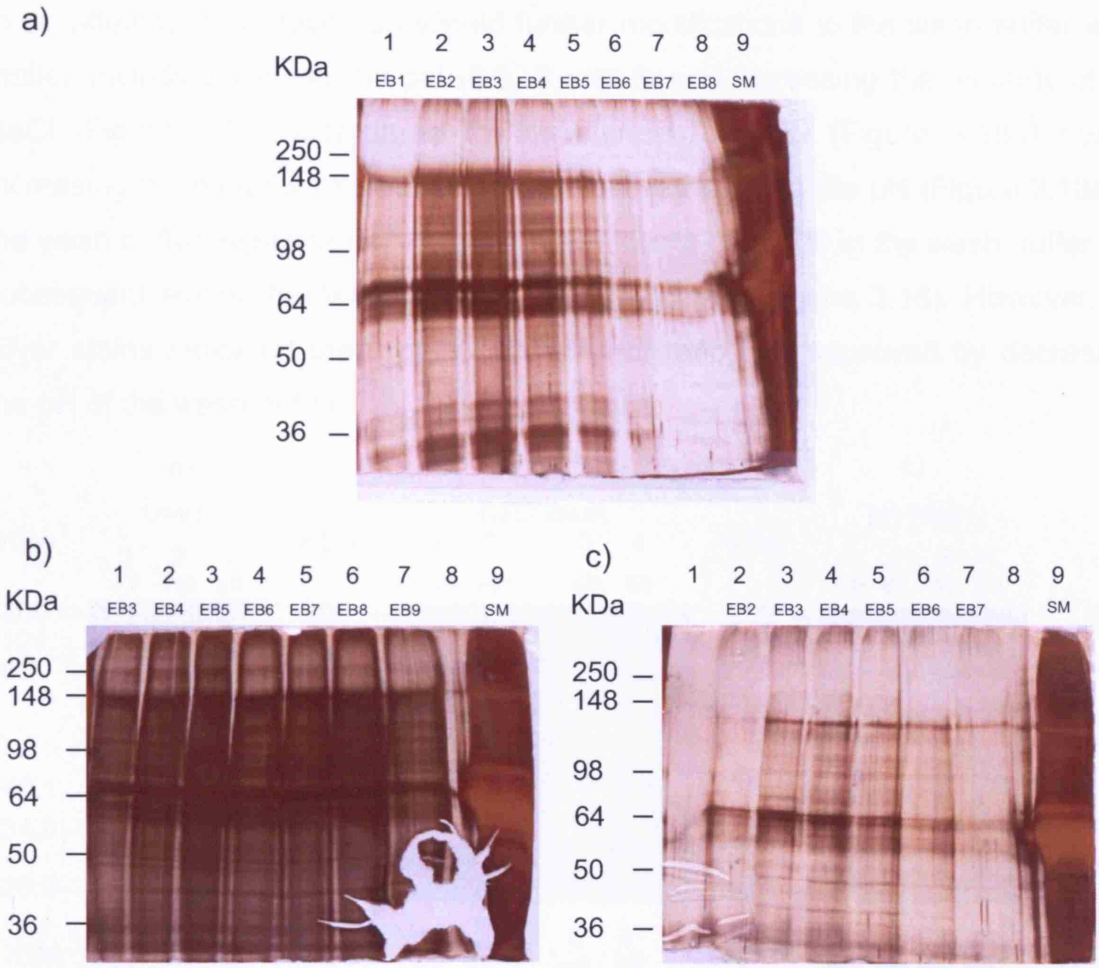
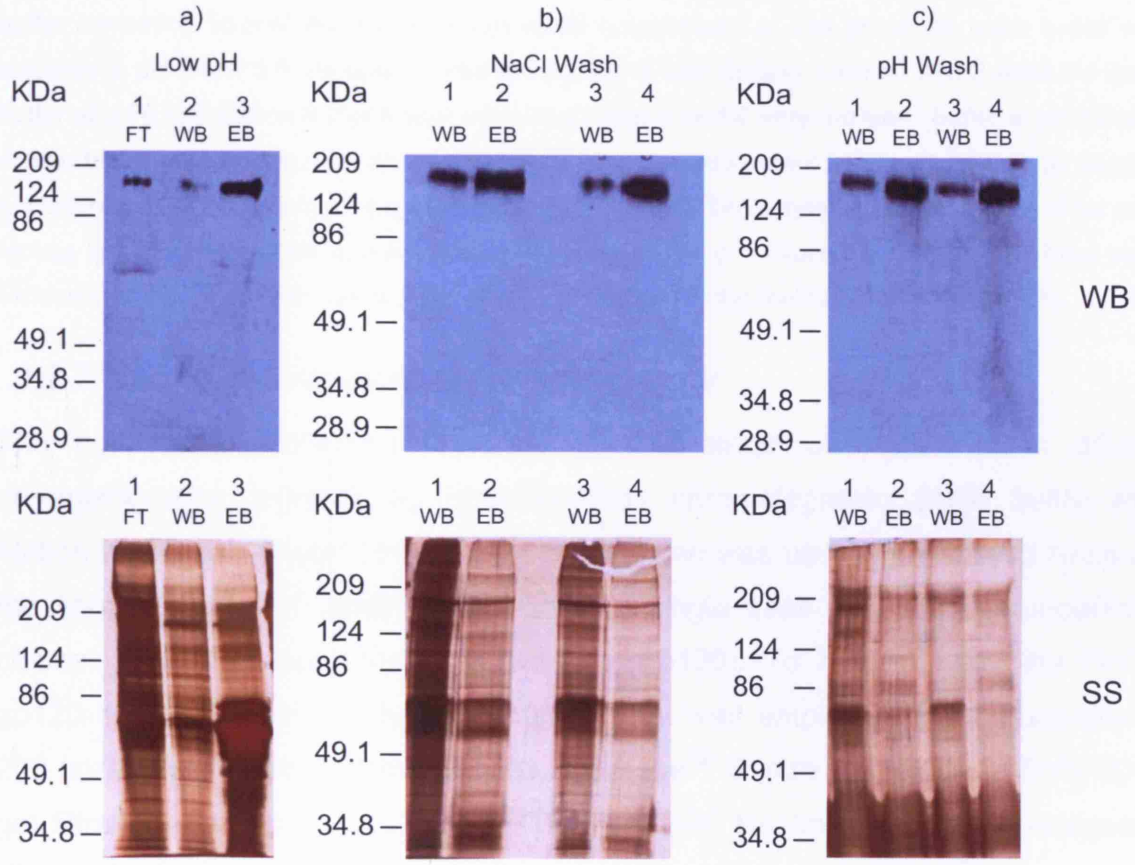


Figure 3.17: Silver Stain analyses of Immunoaffinity Column purification of HIV-2_{ROD} gp120
Elution fractions of immunoaffinity columns, as analysed in Figure 3.16 were screened by silver stain. See Figure 3.16d for information of buffers used in these experiments. 15µl of each fraction was run on a 5-15% PAGE using reducing conditions. **a:** Protein was eluted at pH 2.5, lanes 1→8 correspond to elution fractions 1→8. **b:** Protein was eluted at pH 11.5, lanes 1→7 correspond to elution fractions 3→9. **c:** Protein was eluted at pH 11.5 but the wash buffer contained 1M NaCl, lanes 2→7 correspond to elution fractions 2→7. In lane 9 of all three gels concentrated 39.5 8 TCSN start material was run as a positive control. The silver stain was performed as stated in the methods (Section 2.4.3.1).

In an attempt to improve purity/yield further modifications to the wash buffer were trialled including varying the pH (6.8, 6 and 5) and increasing the molarity of the NaCl (Figure 3.18). Compared to the standard control (Figure 3.18a) neither increasing the molarity of NaCl (Figure 3.18b) nor altering the pH (Figure 3.18c) of the wash buffer, significantly affected the amounts of gp120 in the wash buffer and subsequent elution fractions (Western blots shown in Figure 3.18). However, the silver stains indicated that gp120/contaminant ratio was improved by decreasing the pH of the wash buffer.



d)

	Gel	Lane	Buffer
Normal Wash Buffer	a	2	50mM phosphate 10mM NaCl pH 6.8
Altering NaCl Content	b	1	50mM phosphate 50mM NaCl pH 5.5
Altering NaCl Content	b	3	50mM phosphate 100mM NaCl pH 5.5
Altering pH	c	1	50mM phosphate 10mM NaCl pH 6.0
Altering pH	c	3	50mM phosphate 10mM NaCl pH 5.5

Figure 3.18: Western Blot and Silver Stain analyses of Immunoaffinity purification wash buffer conditions

To investigate which wash buffer was the most effective prior to eluting at pH 2.5 a variety of conditions were examined (**d**). The methodology was the same as that used for the experiment presented in Figure 3.16, except for elutions being collected as one fraction of 20ml. All samples were run on 5-15% PAGE under reducing conditions. All protein concentrations were determined on the *Bio*photometer (Eppendorf, Cat. No. 6131 000.012). **a:** Standard low pH elution with wash buffer containing 10mM NaCl and adjusted to pH 6.8. Lane 1 contains 2µl of flow through (optical density too high to obtain a reading), lanes 2 and 3 contain 1.25µg of total protein in wash buffer and elution buffer respectively. **b:** The NaCl concentration of the wash buffer was adjusted to 50mM and 100mM. Lanes 1 and 2 contain 0.94µg of total protein from the wash buffer containing 50mM NaCl and elution buffer respectively, lanes 3 and 4 contain 0.44µg of total protein from the wash buffer containing 100mM NaCl and elution buffer respectively. **c:** The pH of the wash buffer was adjusted to pH 6 and 5.5. All lanes contained 0.76µg of total protein. Lanes 1 and 2 were the wash buffer at pH 6 and elution buffer respectively and Lanes 3 and 4 were the wash buffer at pH 5.5 and elution buffer respectively. The primary antibody used in western blot (WB) was ARP 3030 used at a dilution of 1:10 incubated at room temperature for 60min. The secondary antibody was Goat anti-Mouse IgG HRP incubated at room temperature for 30min at a dilution of 1:5000. The blots were exposed for 30s. The silver stains (SS) were run as stated in the methods (Section 2.4.3.1).

3.2.7 Size Exclusion Chromatography

The purification procedure identified to this stage was GNA lectin affinity chromatography, followed by Immunoaffinity chromatography (ARP 3085) with elution at low pH. (Note: The Immunoaffinity step was usually performed twice as the flow through and binding buffer from a single pass over the immunoaffinity column often contained residual HIV-2_{ROD} gp120). To further purify the HIV-2 gp120 size exclusion column chromatography was employed. Both Superdex^{TM} 200 (size limit 10KDa to 600KDa) and Superose^{TM} 6 (size limit 5KDa to 5000KDa) gel filtration columns run on an AKTA FPLC (GE Healthcare) were investigated (Figure 3.19).

To compare two gel filtration media it is necessary to determine the partition coefficient (K_{av}) of your protein. K_{av} is a function of the elution volume of a molecule and can be determined by the following equation:

$K_{av}= (V_e-V_o)/(V_t-V_o)$

Where,

V_e = the elution volume

V_o = the void volume

V_t = the total column volume

For purification purposes a K_{av} of approximately 0.5 for the protein of interest is ideal. The K_{av} for HIV-2 gp120 purified on the Superdex 200 column was -2.48 x 10^3 indicating that the protein eluted in the void volume and therefore was not retarded by the column to allow separation. The K_{av} for HIV-2 gp120 purified on the Superose 6 column was 0.3, indicating greater interaction with the column allowing better separation from the contaminants loaded (see Figure 3.19). The presence of HIV-2_{ROD} gp120 in the main peak of the Superose 6 column was confirmed by western blot (Figures 3.19 and 3.20). HIV-2_{ROD} gp120 was detected in fraction B2-B10 which correspond to the main peak centered on 12.5ml and ending on a shoulder (fraction B9 and B10 - Figure 3.20).

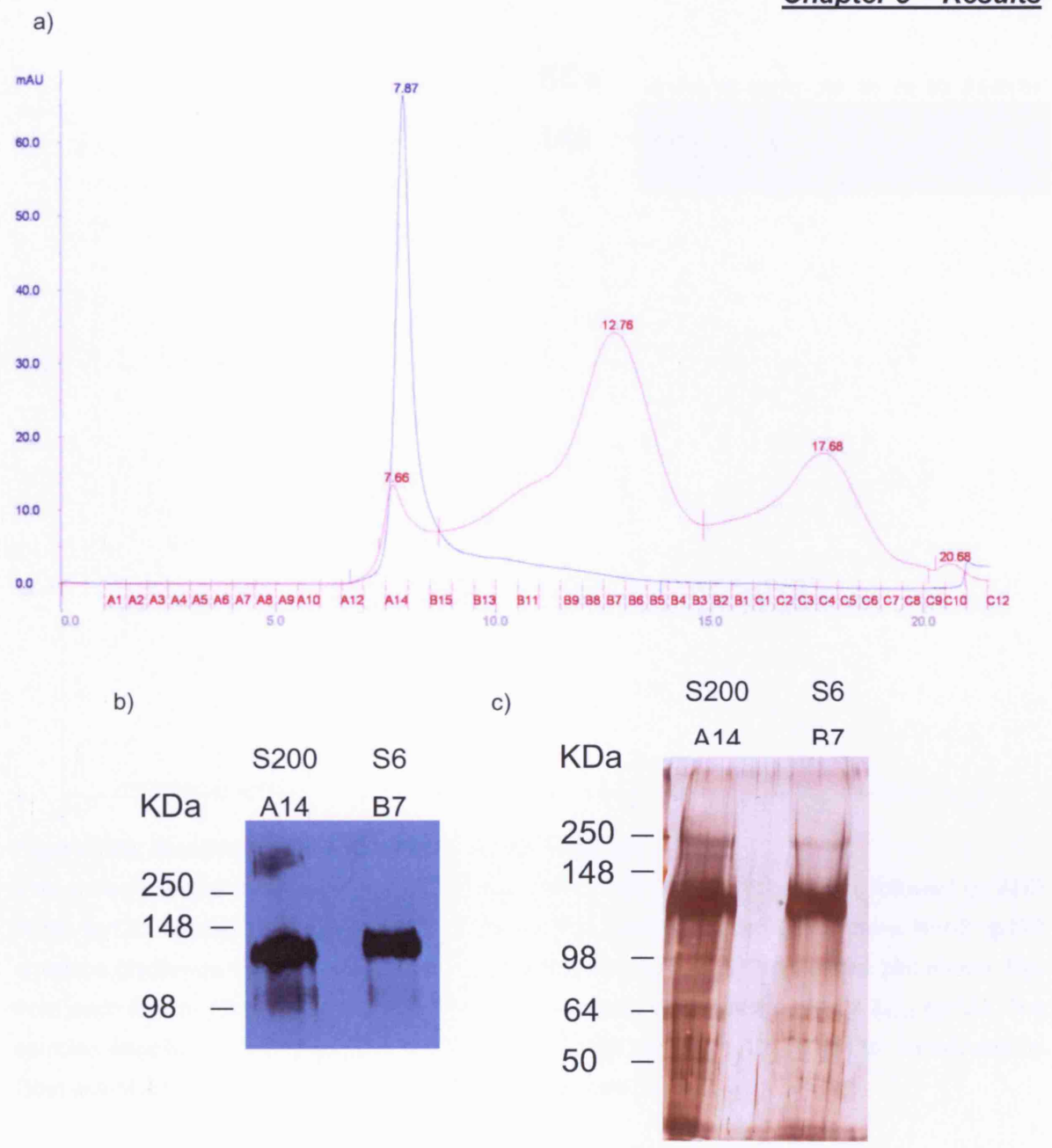


Figure 3.19: Comparison between Superdex 200 and Superose 6 gel filtration

50µg of GNA/ARP 3085 purified HIV-2 gp120 was loaded onto each column in a volume of 100µl with the NaCl concentration adjusted to the appropriate running buffer. **a:** Shown in blue is the trace from the Superdex 200 (S200) column (running buffer 300mM NaCl, 50mM Tris pH 8) and in red is the trace from the Superose 6 (S6) column (running buffer 1M NaCl, 50mM HEPES pH 6.5). The peak containing HIV-2 gp120 from the Superdex 200 was at 7.87ml and the relevant peak from the Superose 6 column centred around 12.76ml. This was confirmed by western blot (**b**) probed with ARP 3030 (1:10 TCSN) and Goat anti-Mouse IgG HRP (1:5000) and exposed for 30s. The relative purity of the peak fractions was assessed by silver stain (**c**) 0.45µg of total protein was loaded for each sample on each gel. All samples were run on 5-15% PAGE under reducing conditions.

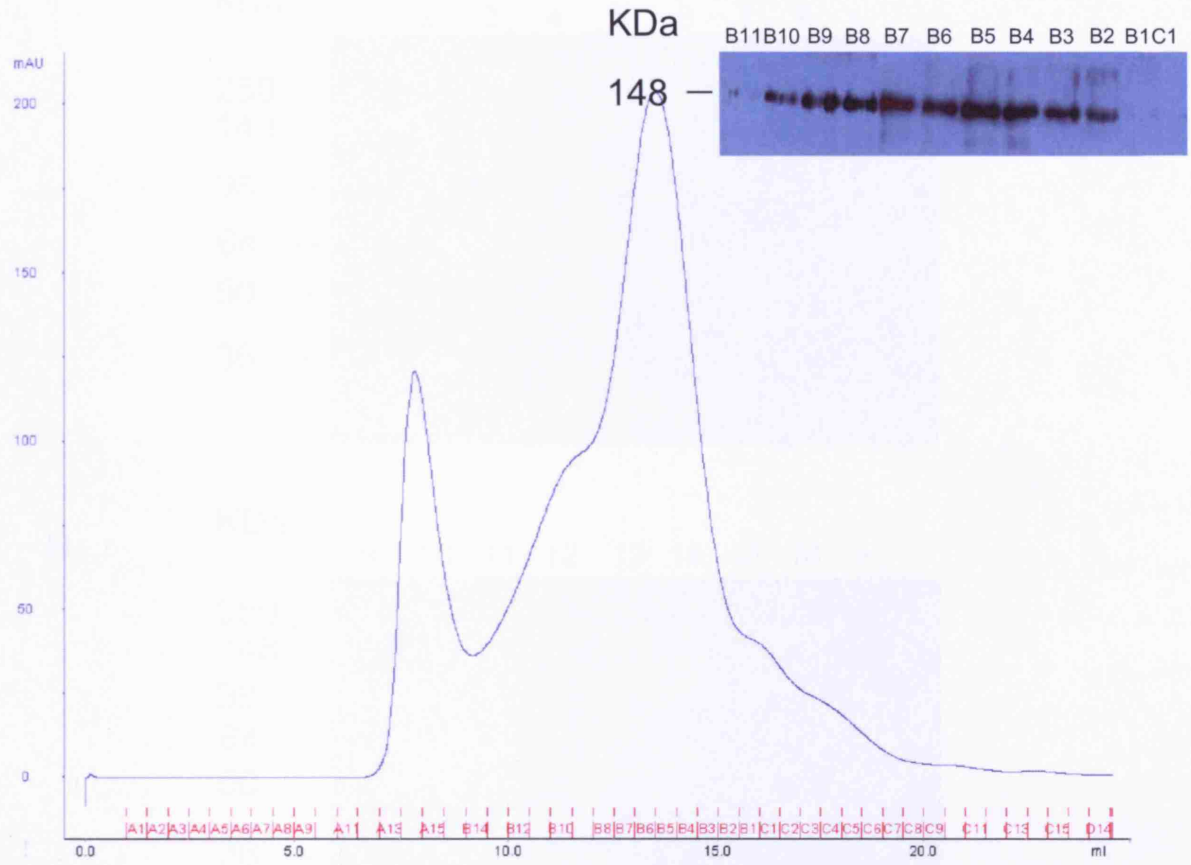
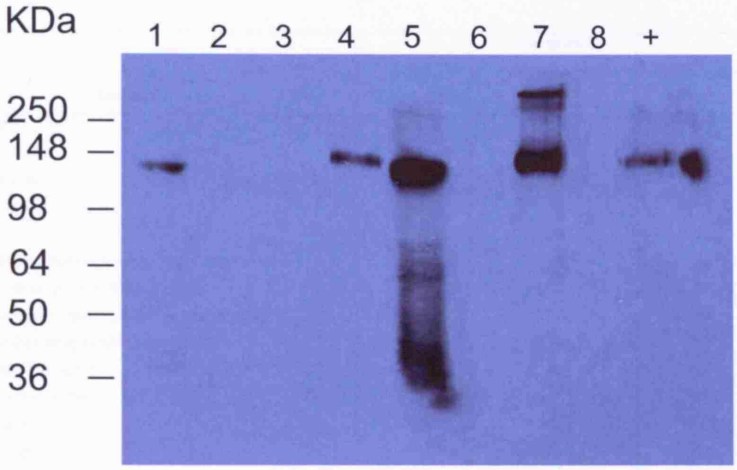


Figure 3.20: Superose 6 Column elution profile of gp120

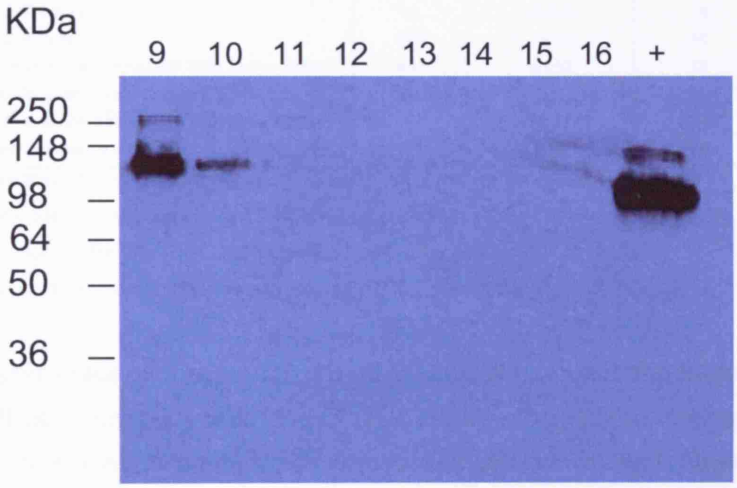
This shows the results of 100µl of concentrated (1000 fold) affinity elution (GNA followed by ARP 3085) gp120 injected over a Superose 6 column. The peak at fraction B5 contains HIV-2 gp120 envelope glycoprotein (Fraction A13 was negative for HIV-2 gp120). The western blot shows 15µl from each fraction (0.5ml) between B11 and C1 indicating the presence of HIV-2_{ROD} gp120. The samples were run on 5-15% SDS PAGE, probed with ARP 3030 at 1:10 (TCSN) for 1h followed by Goat anti-Mouse IgG HRP (1:5000) for 30min and exposed for 2s.

Figure 3.21 shows a series of western blots monitoring the established purification protocol, together with a table (Figure 3.21d) indicating the source of the sample run in each lane. Lane 19 (Figure 3.21) shows a smaller band running at approximately 80KDa this could possibly be the result of cleavage in the V3 loop of HIV-2 gp120 (Clements et al., 1991), but this band appears to be removed on gel filtration.

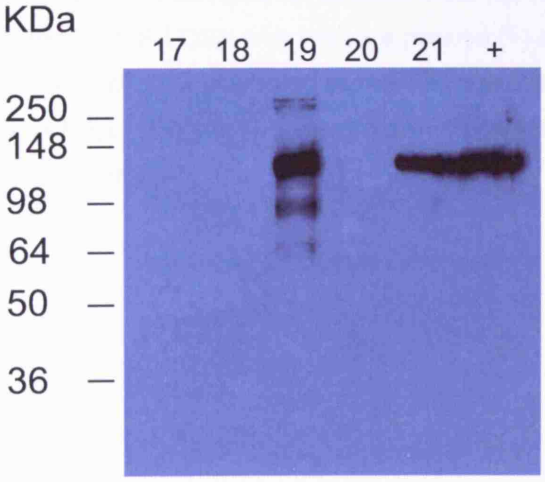
a)


KDa
1 2 3 4 5 6 7 8 +
250
148
98
64
50
36

b)


KDa
9 10 11 12 13 14 15 16 +
250
148
98
64
50
36

c)


KDa
17 18 19 20 21 +
250
148
98
64
50
36

d)

Western Blot No.	Sample	Total Volume/ml	Total protein concentration/ mg/ml	Volume used in WB /µl	Equivalent to µg[1]	Western Blot Result
1	Start Material	100	N/D	18	N/K	+
2	GNA Flow Through	100	N/D	18	N/K	-
3	GNA Wash Buffer	200	0.617	2.1	1.3	-
4	GNA Elution Buffer	250	0.023	18		+
5	GNA Resin		N/D	18		+
6	Flow Through from concentrating GNA Elution Buffer[2]	250	0.004	18		-
7	Concentrated GNA Elution Buffer	3	1.139	1.1	1.3	+
8	Flow Through from Desalting GNA Elution Buffer[3]	2.5	0.002	18		-
9	Elution Buffer Desalting GNA Elution Buffer	3	0.708	1.8	1.3	+
10	ARP 3085 Flow through 1	2.5	0.072	18	1.3	+
11	ARP 3085 Binding Buffer 1	50	0.013	15.2	0.2	-
12	ARP 3085 Wash Buffer 1	50	0.003	15.2		-
13	ARP 3085 Elution Buffer 1	200	0.003	18		-
14	ARP 3085 Flow Through 2	50	0.013	15.2	0.2	-
15	ARP 3085 Binding Buffer 2	50	0.003	18		-
16	ARP 3085 Wash Buffer 2	50	0.000	18		-
17	ARP 3085 Elution Buffer 2	200	0.000	18		-
18	Flow Through from concentrating ARP 3085 Elution Buffer[4]	400	0.002	18		-
19	Concentrated ARP 3085 Elution Buffer	0.1	0.6	2.2	1.3	+
20	Flow through from concentrating ARP 3085 Elution Buffer[3]	4	0.011	18	0.2	-
21	B7 fraction from Superose 6 column	0.5	0.03	6.6	0.2	+

[1] Blank spaces indicate that OD readings measured by were not significantly above zero.

[2] The elution buffer was concentrated using Vivacell 70 (see section 2.3.1.3)

[3] Desalting the GNA elution buffer into ARP 3085 binding buffer (see section 2.3.3)

[4] The elution buffer was further concentrated using Vivaspin 500 (see section 2.3.1.3)

Figure 3.21: Western blot of fractions from all stages of the established purification protocol
The working purification protocol consisted of GNA Lectin affinity chromatography followed by ARP 3085 immunoaffinity chromatography (with two passes of GNA purified material) followed by size exclusion chromatography on a Superose 6 column. All samples were run on 5-15% PAGE using reducing conditions. For information of samples on western blots (**a**), (**b**) and (**c**) see table **d**. In the right hand lane of all three gels 39.5 8 TCSN was run as a positive (+) control. The primary antibody was ARP 3030 used at a dilution of 1:10 incubated at room temperature for 60min. The secondary antibody was Goat anti-Mouse IgG HRP incubated at room temperature for 30min at a dilution of 1:5000. The blots were exposed for 30s.

3.2.7.1 Calculation of Purification Losses

To determine the efficacy of the purification process an ELISA was developed. 1.4×10^{-5} mg/ml of my purified HIV-2_{ROD} gp120 was sequentially two fold diluted to 8.8×10^{-7}mg/ml and these dilutions were used to generate a standard curve on which to base estimates of total gp120 in start material (Figure 3.22). Typical results showed that 1l of TCSN produced 1.3mg of protein and the purification process yielded 275µg (21%).

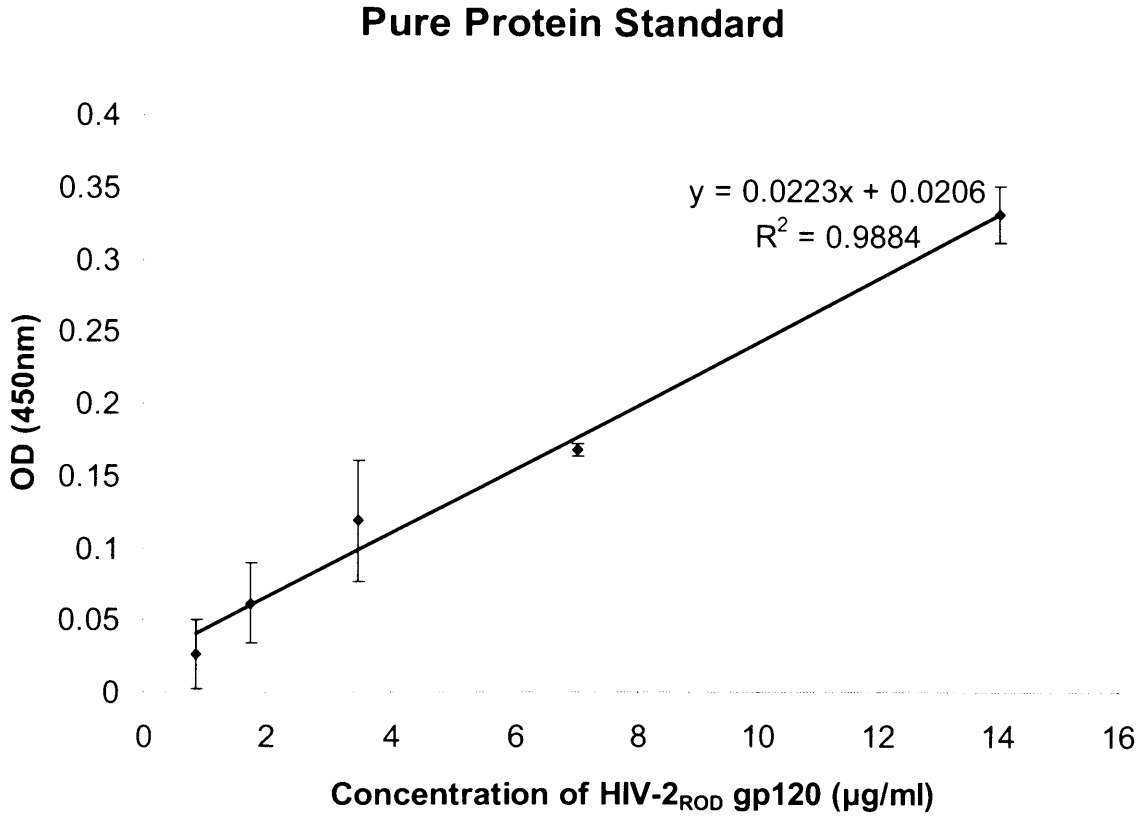


Figure 3.22: Standard Curve for ELISA determination of HIV-2 gp120 concentration.
The concentration of the pure HIV-2 gp120 was read at 280nm using a Beckman (USA) Du® 640 Spectrophotometer (Section 2.4.1). A two fold dilution series (in sensitising buffer 75mM $NaHCO_3$, 25mM Na_2CO_3 made up to 100ml with DDW at pH 9.5), carried out in triplicate, of the HIV-2 gp120 was utilised to produce a standard on which to base estimates of total gp120 in the original TCSN and all stages of the purification process. The ELISA is described in the methods chapter (Section 2.4.5).

Further ELISAs (not shown) indicated that for both the GNA and Superose 6 columns almost 100% of the protein loaded was recovered. The apparent retention of gp120 on the GNA resin (Figure 3.21 lane 5) may relate to the column being used multiple times and a build up of non-specific binding to the resin which is not removed from the resin by the elution buffer. Similarly, analyses of the flow throughs, binding buffers and wash buffers of the ARP 3085 column showed negligible losses of HIV-2 gp120 at these stages. Therefore, the bulk of HIV-2 gp120 loss must have been due to irreversible binding to the ARP 3085 column. Such binding was antibody related as no non-specific binding to the column resin was observed (results not shown). As well as the standard elution conditions (250ml of 50mM Glycine, 10mM NaCl at pH 2.5) a variety of others, (i) a further 250ml of standard elution buffer, (ii) a further 250ml of standard elution buffer containing 0.5M NaCl, (iii) elution with an organic solvent (50% ethylene glycol pH 11), gave no improvement in recoveries from the immunoaffinity column.

3.2.8 Conclusion

Experiments undertaken during this developmental phase indicated that neither metal-ion affinity (Nickel/Cobalt) nor ion exchange chromatography (See Figure 3.9) were suitable first steps in a successful purification process. This was due to poor affinity and sample heterogeneity (glycosylation) respectively. The results shown here (Section 3.2.3) revealed that there are very real differences between lectin specificities even when they are reportedly all capable of binding the same types of sugar moieties (α-linked mannose). The outcome of testing these different lectins yielded the first purification step in the form of *Galanthus nivalus* (GNA) lectin affinity chromatography (Figure 3.15). Subsequent ELISA's showed that this step gave very close to (if not) 100% recovery of HIV-2_{ROD} protein and reduced the amount of contaminants considerably (see Section 3.2.5.1). However, none of the lectins tested were able to purify construct 1 (further discussed in Section 4.2). Therefore, at this stage this construct was set aside in favour of the GNA purified construct 39.

At this point the HIV-2_{ROD} protein was not of a suitable purity for crystallisation trials or other structural and functional investigations. Therefore, a further purification step was required and the most likely candidate was considered to be antibody affinity chromatography. Following a thorough investigation (Table 3.3) of the antibodies that had been purified from hybridoma supernatant (NIBSC), ARP 3085 was selected for affinity purification. ARP 3085 was chosen because it bound construct 39 in a range of different native conditions indicated by kinetic data from BIAcore assays, the results of immuno-precipitation and native gels. However, subsequent ELISA results indicated this step in the purification procedure gave the greatest losses reducing the yield of protein from start material to approximately 20%.

A further purification step was required in order to obtain protein of a quality suitable for crystallisation trials. Size exclusion chromatography was assessed and a comparison of Superdex 200 and Superose 6 columns (section 3.2.5) showed Superose 6 to provide the greater separation from the remaining contaminants. This step also allowed the protein to be buffer exchanged into high salt which we knew from the HIV-1 gp140 studies in the laboratory (Billington *et al.,* unpublished) delayed aggregation. This step yielded almost 100recovery of the HIV-2_{ROD} protein loaded onto the column.

Final selection of the appropriate fractions for crystallisation trials was decided using dynamic light scattering. Those fractions containing product of the expected molecular weight and the lowest polydispersity were chosen and pooled.

Although, I was unsuccessful in determining a purification procedure for construct 1, a protocol was developed for construct 39 which produced HIV-2_{ROD} protein in a quantity and quality sufficient for initial characterisation of the protein and the setting up of crystallisation trials.

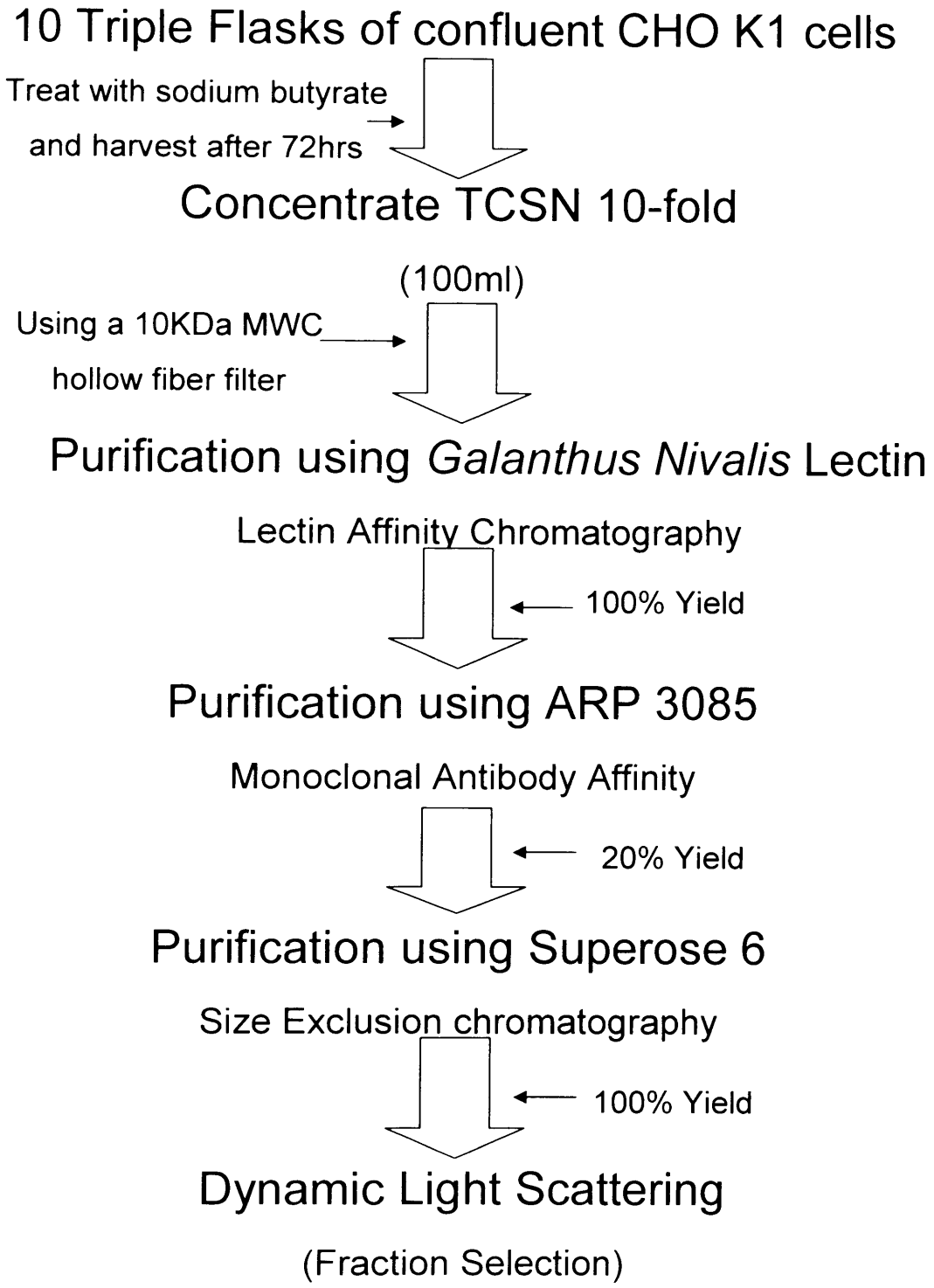


Figure 3.23 - Flow chart of Optimised Purification Protocol

This flow chart indicates the major steps in the purification protocol optimised during this PhD. It also indicates the average potential yield of protein at each step.

3.3 HIV-2_{ROD} gp120 characterisation

3.3.1 Aim

Characterisation of the HIV-2_{ROD} protein was required to determine whether the protein was functional, in respect to binding of conformation dependant antibodies and the cellular receptor CD4, the oligomeric state of the protein and its secondary structure. This would involve the use of several biochemical and biophysical techniques and indicate whether I had produced/purified a correctly folded protein suitable for crystallisation trials.

All characterisation of the HIV-2 gp120 envelope glycoprotein was carried out for construct 39.5 8 (gp120FIB cs S: see Table 3.2). The purified gp120 was stored in a buffer at pH 6.5 containing 1M NaCl and 50mM HEPES.

3.3.2 Assessment of Functionality of Purified gp120

CHO-cell produced gp120 was reactive with conformation-specific MAbs (Table 3.3), suggesting that it was correctly folded. To assess this further, the functionality of gp120 was determined in respect of CD4 binding.

3.3.2.1 CD4 Binding ELISA

An ELISA based on binding CD4 to a plate, followed by binding of a dilution series of HIV-2_{ROD} gp120 and detection of bound gp120 with a monoclonal antibody was employed (Figure 3.24). gp120 clearly bound to CD4 in a concentration dependant manner. Attempts to use baculovirus produced HIV-2_{ROD} gp105 (EVA621 – CFAR) as a control for this assay were unsuccessful as this protein did not bind to the immobilised CD4 used.

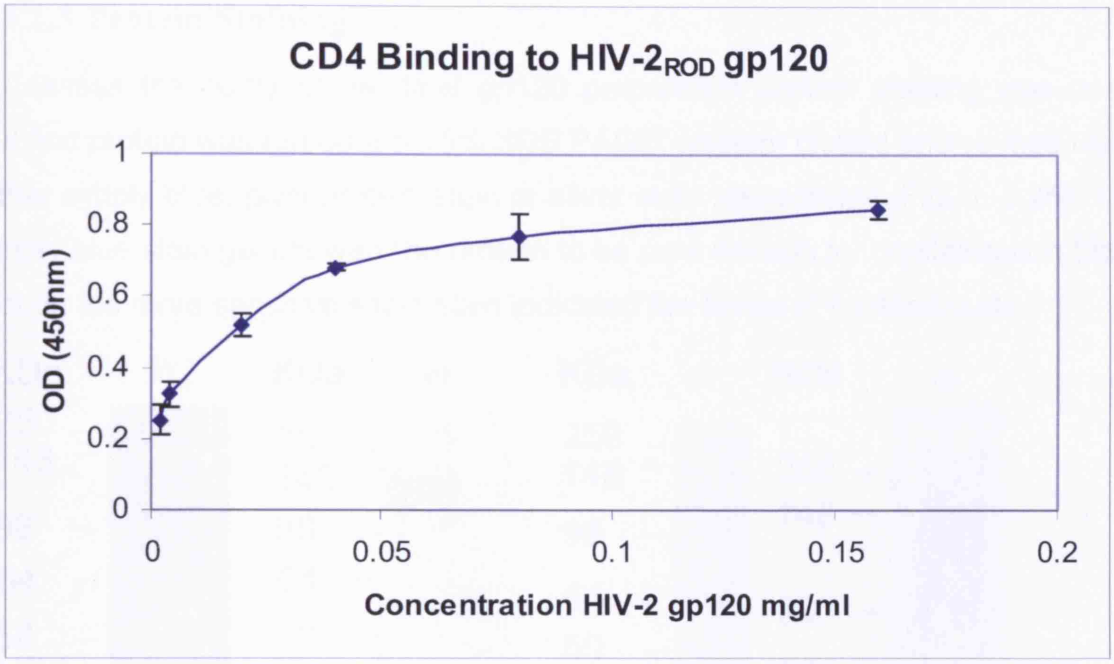


Figure 3.24: CD4 Binding to HIV-2_{ROD} gp120

Wells of a 96-well plate were coated with 0.25µg of 2 Domain CD4 (NIH Cat. No. 7356) and reacted with 50µl aliquots of a two fold dilution series of pure HIV-2 gp120 (in diluting buffer: 0.5g Marvel, 50µl Tween 20 made to 100ml with PBS). Bound gp120 was detected with primary antibody ARP 3030 (50µl) used at a concentration of 0.02mg/ml followed by secondary antibody, Goat anti-Mouse IgG HRP, used at a dilution of 1:1000. OD_{450} values were determined for three replicates at each dilution of HIV-2 gp120 and an average of negative control wells (these wells did not contain CD4 and were blocked with Marvel before addition of HIV-2 gp120 and produced an average OD_{450} value of 0.065) removed from these values. Following this standard deviation was calculated for the replicates.

3.3.2.2 Surface Plasmon Resonance: BIAcore

A determination of the binding affinity (K_D) of HIV-2 gp120 envelope glycoprotein for CD4 should have been possible using surface plasmon resonance, BIAcore. However, despite varying the assay in respect of, (i) binding CD4 to the CM5 chip and passing gp120 over and vice versa, (ii) using a range of protein concentrations, and (iii) using a variety of buffer systems, reproducible K_D estimates were not obtained. CD4 binding affinity of HIV-2 Env has been reported by others, to be in the range 4.5×10^{-8} - 7×10^{-8}M (gp105-gp140), some 10 to 25 times lower than HIV-1 Env affinity for CD4 (Bahraoui et al., 1992; Moore, 1990).

3.3.2.3 Protein Staining

To assess the purity of the final gp120 preparation protein staining was used. Purified protein was run on a 5-15% SDS PAGE, western blotted and stained using either simply blue, glycoprotein stain or silver stain respectively (Figure 3.25). The simply blue stain gel showed the protein to be pure enough for crystallisation trials, though the more sensitive silver stain indicated low levels of contaminants.

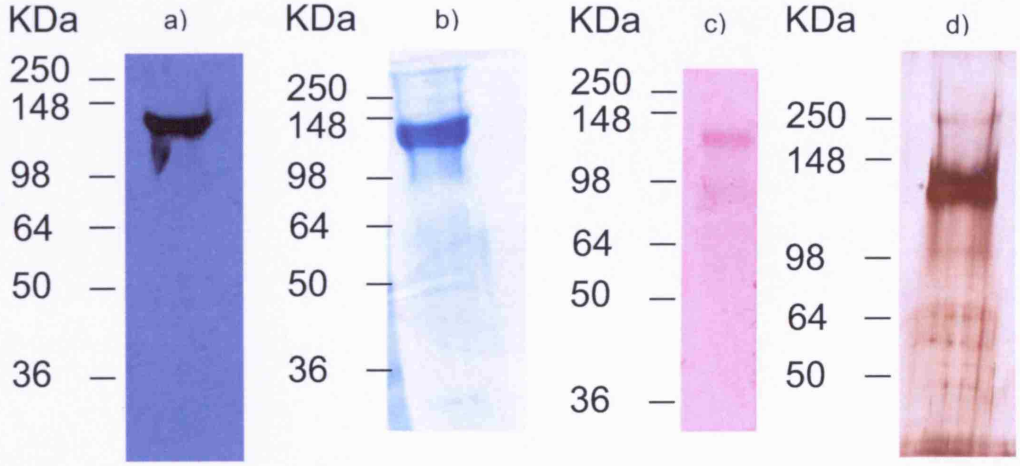


Figure 3.25: Assessment of purity of HIV-2 gp120

HIV-2 gp120 positive elutions from a Superose 6 size exclusion column were pooled and concentrated (40 fold) using a spin filter (30KDa cut off). Samples were run on 5-15% PAGE under reducing conditions. **a:** 125ng was western blotted and detected using ARP 3030 (1:10 TCSN) incubated for 1h followed by Goat anti-Mouse IgG HRP (1:5000) for 30min and exposed for 30s. **b:** A simply blue stain of 10µg of HIV-2 gp120 envelope glycoprotein. **c:** A glycoprotein stain of 225ng of HIV-2 envelope glycoprotein. **d:** A silver stain of 125ng of HIV-2 envelope glycoprotein. Due to the detection limits of the various stains different quantities of HIV-2 gp120 were used.

3.3.2.4 Isoelectric Focusing

To determine the charge heterogeneity of the purified gp120 isoelectric focusing by 2D electrophoresis was used. The isoelectric points predicted from the HIV-2 gp120 sequence by computer programs were weakly basic with values of 7.937 (Sednterp -http://www.cauma.uthscsa.edu/software (Cole and Hansen, 1999)) and 7.77 (ProtParam - http://nimri.nimr.mrc.ac.uk/webselect/ifmolbio.htm). 15µl of TCSN containing HIV-2 gp120 was run on an Immobilline DryStrip pH 3-10 Isoelectric Focusing gel (Amersham Biosciences, Cat. No. 17-6001-12) and then transferred to a 10% PAGE which was subsequently western blotted, using ARP 3030 (at 1:10 Hybridoma TCSN) to detect the products (Figure 3.26a). This

showed the gp120 to have migrated as a smear, 2-23mm from the anode. Using the supplied pH gradient profile (Figure 3.26b), this equated to a pI covering the range pH 3.8 - 5.2. This is in agreement with reports in the literature that the pI of *Pichia pastoris* produced gp105 was 5.2 (Zhang et al., 2002), with the broader range observed here presumably being due to the more complex glycosylation system of mammalian cells (CHO K1) compared to yeast.

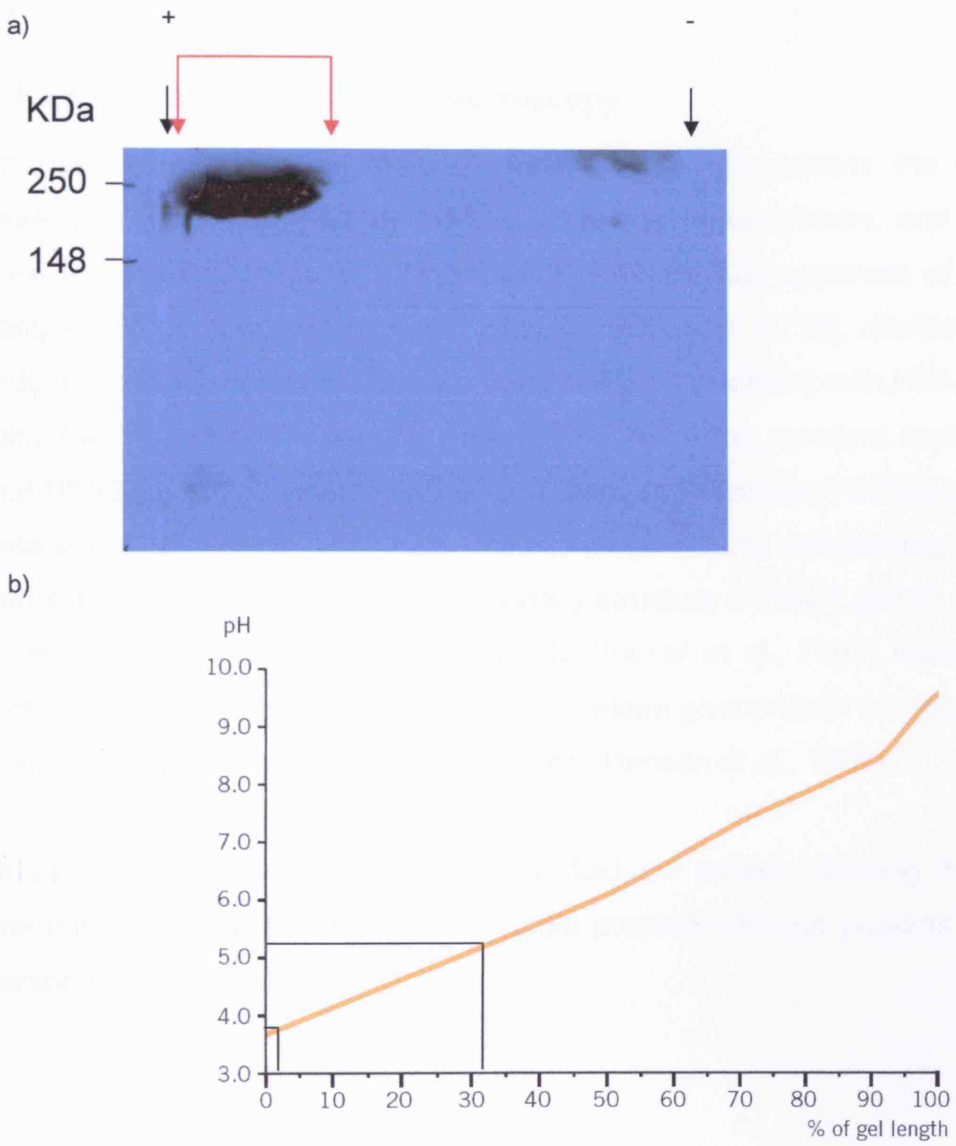


Figure 3.26: Isoelectric focusing of purified gp120

15µl of TCSN containing HIV-2_{ROD} gp120 was run on an Immobilline DryStrip pH 3-10 (Amersham Biosciences, Cat. No. 17-6001-12), 7cm, Isoelectric focusing gel and subsequently transferred onto a 10% PAGE (see Methods Section 2.4.3.6). **a:** Western blotting was performed using ARP 3030 as a primary antibody at a dilution of 1:10 (TCSN) incubated for 1h followed by Goat anti-Mouse IgG HRP secondary antibody incubated for 30min and exposed for 2min. The black arrows indicate the anode (+) and cathode (-) of the 7cm pH 3-10 DryStrip gel. The red arrows indicate the ends of the smear which is between 2mm-23mm (2.9%-32.9%) from the positive end of the gel. **b:** The figure shows the pH gradient profile for the Immobilline DryStrip pH 3-10 (7cm) provided by Amersham Biosciences. HIV-2 gp120 produced a smear between 2.9% and 32.9% of the gel, from this it could be calculated that the pI of gp120 was between 3.8 and 5.2.

3.3.3 Folding

3.3.3.1 Circular dichroism spectroscopy

To investigate the folded state of the envelope glycoprotein, the soluble gp120 molecules were analysed by circular dichroism spectrometry, and the data was collected at 20°C. Figure 3.27 shows the far-UV CD spectrum of a 10x diluted sample (Stock concentration = 0.73mg/ml [OD_{280} = 1.738], diluted with DDW to reduce the NaCl content to 0.1M). The profile is consistent with HIV-2 gp120 being folded with a low alpha helical content. Analysis using standard methods suggests that HIV-2_{ROD} gp120 (construct 39) contains (approximately) 22% alpha helix, 24% beta-sheet, 23% turn, and 29% unstructured. This is comparable with predicted and experimentally determined secondary structure of HIV-1 gp120 (Hansen et al., 1996). Other groups (Chen et al., 2000; Sourial et al., 2005) have used circular dichroism to study their HIV-2 and SIV envelope glycoprotein constructs and stated that it is comparable with those of Hansen (Hansen et al., 1996).

Heating the sample to 85°C did not unfold the protein showing that it has high thermal stability, which is not an unusual property of large proteins (Dr S. Martin, personal communication)

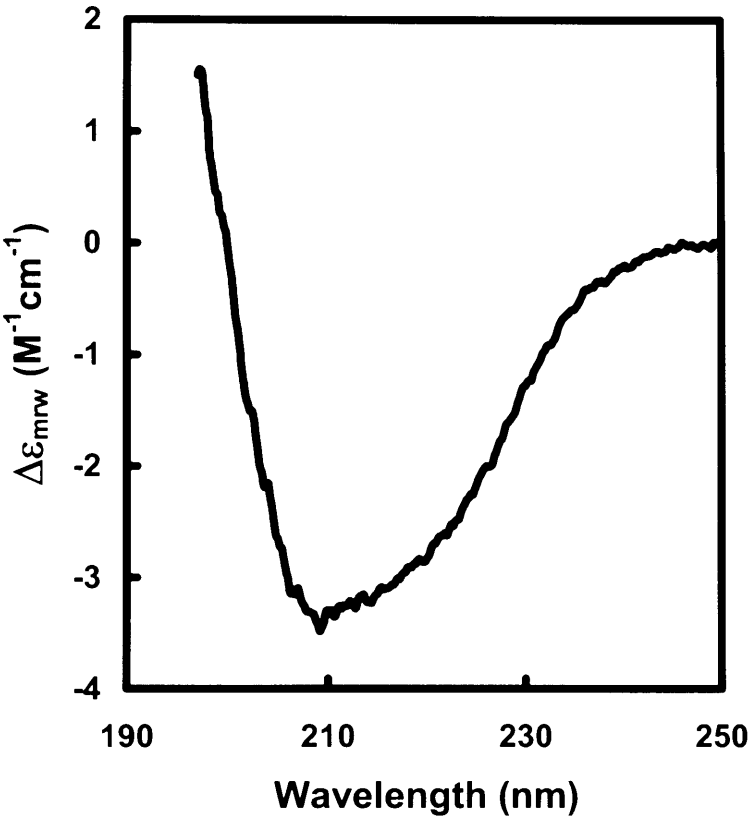


Figure 3.27: Far-UV spectrum of HIV-2_{ROD} gp120

Purified and concentrated HIV-2_{ROD} gp120 was diluted 10 fold (to dilute out the 1M NaCl, which may interfere with the spectrum) to give a concentration of 0.073mg/ml, 200µl of which was used to produce this spectrum. Analysis using standard methods suggests that HIV-2_{ROD} gp120 (construct 39) contains (approximately) 22% α-helix, 24% β-sheet, 23% turn, and 29% unstructured.

3.3.3.2 Fluorescence spectroscopy

A further method employed to assess the folded nature of the HIV-2 envelope glycoprotein was fluorescence spectroscopy. Figure 3.28 shows the fluorescence spectrum of the HIV-2 gp120 (1:10 diluted sample of 0.73mg/ml). The emission maximum was at 345nm. Free tryptophan emits at 356nm, hence some of the tryptophans (25 in construct 39.5 8) must be buried, again suggesting that HIV-2_{ROD} gp120 is folded.

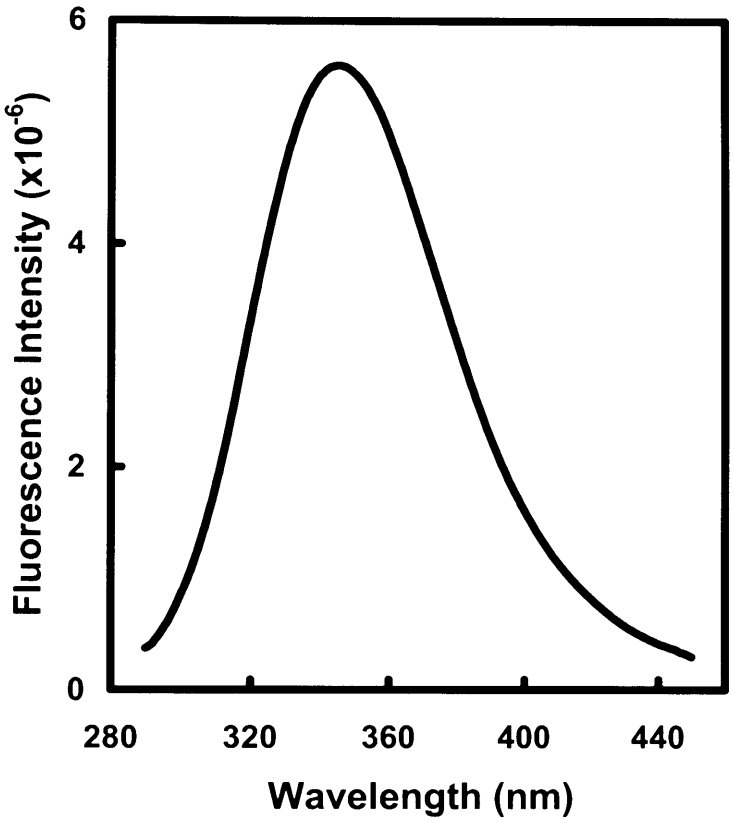


Figure 3.28: Fluorescence spectrum of HIV-2_{ROD} gp120

The same sample used to produce Figure 3.27 was used here. The emission maxima of 345nm suggests that the protein's tryptophans are buried and that the protein is therefore folded.

3.3.4 Oligomeric State

3.3.4.1 Native PAGE

To assess the oligomeric structure of the purified gp120 Native PAGE was used (Figure 3.28). Detection of gp120 by either western blot or simply blue staining indicated that the protein was a large oligomer running above the 669KDa marker.

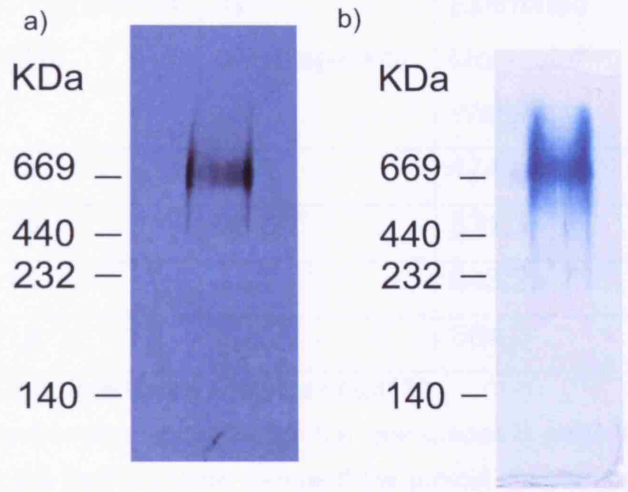


Figure 3.29: Native PAGE analysis of gp120

HIV-2 gp120 positive elutions from a Superose 6 size exclusion column were pooled and concentrated (40 fold) using a spin filter (30KDa cut off). Samples of purified HIV-2 envelope glycoprotein were run on 4-12% native gels. **a:** Western blot of 125ng of HIV-2_{ROD} gp120 detected using ARP 3030 at 1:10 (TCSN) incubated at room temperature for 1h followed by Goat anti-Mouse IgG HRP at 1:5000 incubated at room temperature for 30min. The blot was exposed for 30s. **b:** Simply blue stain of 10µg of HIV-2 envelope glycoprotein.

3.3.4.2 Dynamic Light Scattering

To determine which Superose^{TM} 6 fractions to use for structural and functional studies dynamic light scattering analysis was carried out on every fraction in the peak known to contain HIV-2 gp120 (Figure 3.20). For each fraction 10 individual readings were taken and the average was calculated from which the hydrodynamic radius and the percentage polydispersity were automatically calculated. From these estimates of the molecular weight of the species in the cuvette were made. The example given below is typical of the readings obtained (Table 3.4). Polydispersity values of <30% are indicative of protein suitable for structural analysis (D'Arcy, 1994; Ferre-D'Amare and Burley, 1997), whilst this estimated molecular weight range (474.8-684.6) was indicative of at least a tetramer.

Sample*	Concentration mg/ml	% polydispersity	Estimated Molecular Weight	SOS Noise
B4	0.13	22.1	474.8	6.04
B5	0.16	24.6	531.3	4.69
B6	0.15	23.4	643.2	3.71
B7	0.12	23.1	684.6	4.03

Table 3.4: Dynamic Light Scattering analysis of gp120

Results are based on a monomodal distribution (i.e. one species is present), the molecular weight is an estimate based on the hydrodynamic radius of the protein species present in the sample. The percentage polydispersity indicates the distribution of individual molecular weights. SOS Noise is a further indication of the purity of the sample, samples with a SOS Noise value <5 indicates negligible error or low noise, whereas a value >20 indicates a high degree of error is likely in the other calculated values. *Fraction number from a Superose 6 column (see Figure 3.20).

3.3.4.3 Analytical Ultracentrifugation

To more precisely assess the oligomeric state of HIV-2 gp120 both equilibrium and velocity analytical ultracentrifugation were employed. Dr J. Eccleston (Division of Physical Biochemistry at NIMR) assisted with the analysis.

3.3.4.3.1 Equilibrium Analytical Ultracentrifugation

Based on the amino acid and estimated carbohydrate content (Leonard et al., 1990) of 39.5 8 gp120 a partial specific volume of 0.668 was calculated by the programme Sednterp (http://www.cauma.uthscsa.edu/software) (Cole and Hansen, 1999). Additionally the density of the gp120 sample buffer (1M NaCl, 50mM HEPES at pH 6.5) was calculated to be 1.04g/ml. These values were applied to the analysis of the equilibrium data.

Nine sets of data were collected employing three protein concentrations and at three speeds of centrifugation. Figure 3.30 shows the raw data for the nine sets, of equilibrium results together with data collected at 726 x g where no equilibrium was achieved.

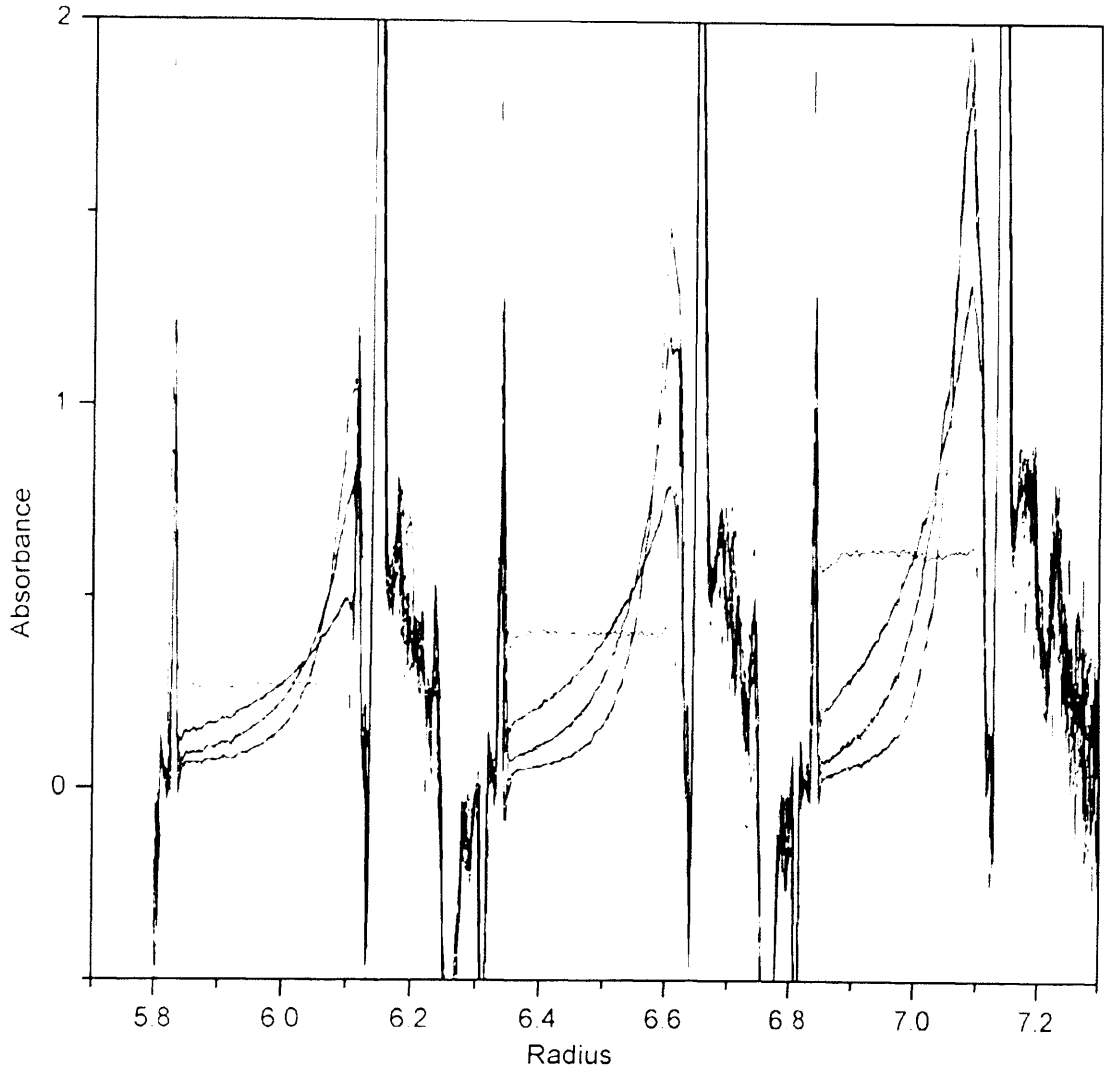


Figure 3.30: Equilibrium Analytical Ultracentrifugation Raw Data for gp120

Three samples of purified HIV-2 gp120, with optical densities of 0.24, 0.36 and 0.57 at 280nm, were centrifuged at speeds of 2016, 4536 and 8064 x g on a Beckman XL-A analytical ultracentrifuge at 20°C. The data shown indicates equilibrium was reached at all speeds and concentrations. The trace for centrifugation at 726 x g, where equilibrium was not achieved, are also shown (the three horizontal lines shown at each speed).

The nine data sets were fitted to a single species model (Figure 3.31). Individual data sets showed residuals (deviation from the line of best fit) between the data and the fit of between 0.04 and -0.04 with some systematic deviation indicating a more complex situation. However, based on a single species model, the molecular mass was determined to be 315.3KDa, which is comparable with a trimeric state of gp120.

Figure 3.31: Nine Equilibrium data sets fitted to a single species model for gp120
Left to right shows optical densities of 0.24, 0.36 and 0.57 at 280nm. Top to bottom shows speeds of 2016, 4536 and 8064 x g. Within each trace the line of best fit residuals are shown.

3.3.4.3.2 Velocity Analytical Ultracentrifugation

The method by which the equilibrium analytical centrifugation data was analysed was limited to a single species model. There was systematic deviation between the residuals and fit of the nine data sets, indicating possible sample heterogeneity. Analysis of velocity analytical centrifugation by the more complex method of SEDFIT (Schuck, 2000) allows for sample heterogeneity. Figure 3.32 shows the raw data from the velocity analytical centrifugation.

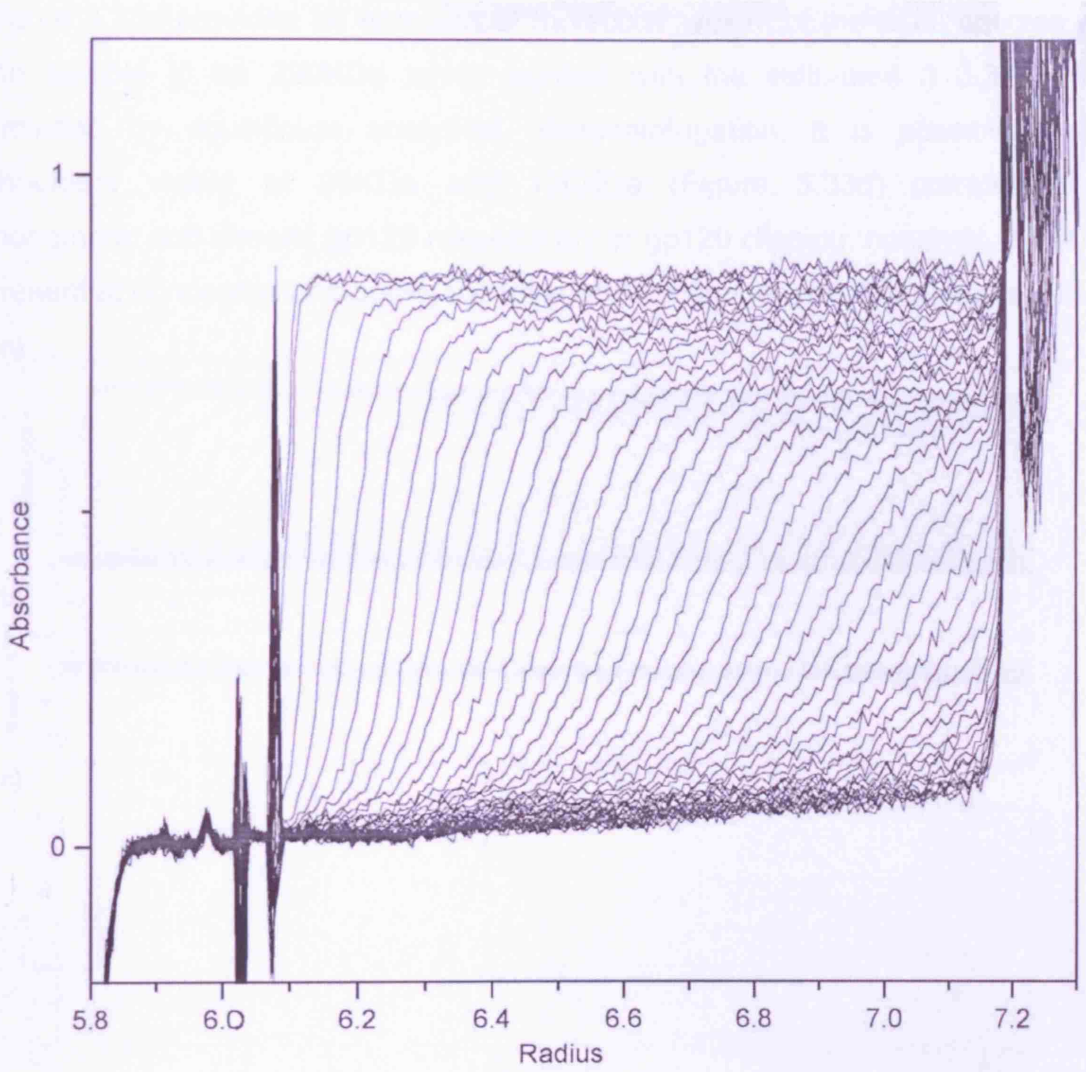


Figure 3.32: Velocity Analytical Ultracentrifugation Raw Data for gp120

Purified HIV-2 gp120 protein, with an optical density of 1 (280nm), was centrifuged at a speed of 129,024 x g and readings were taken in a stepwise fashion (0.01cm) at a wavelength of 280nm.

This data was analysed by the transport method (using Beckman's software) which assumes a single species to be present. The data was calculated to have a sedimentation coefficient of 9.19s.

The data was then further analysed by a more powerful programme called SEDFIT (Schuck, 2000) that fits the data to a distribution of s values so that different molecular weight components can be identified (Figure 3.33). Figure 3.33c indicated that the sedimentation coefficient of HIV-2 gp120 was 10s which concurred with the value of 9.19s provided by the Transport method of analysis.

Figure 3.33d provides an estimate of molecular weight of the main species within the sample to be 290KDa which agreed with the estimated 315.3KDa result provided by equilibrium analytical ultracentrifugation. It is possible that the shoulders visible at 90KDa and 190KDa (Figure 3.33d) corresponded to monomeric and dimeric gp120 respectively or gp120 clipping, however, these were present at concentrations below the level of detection for western blot analysis.

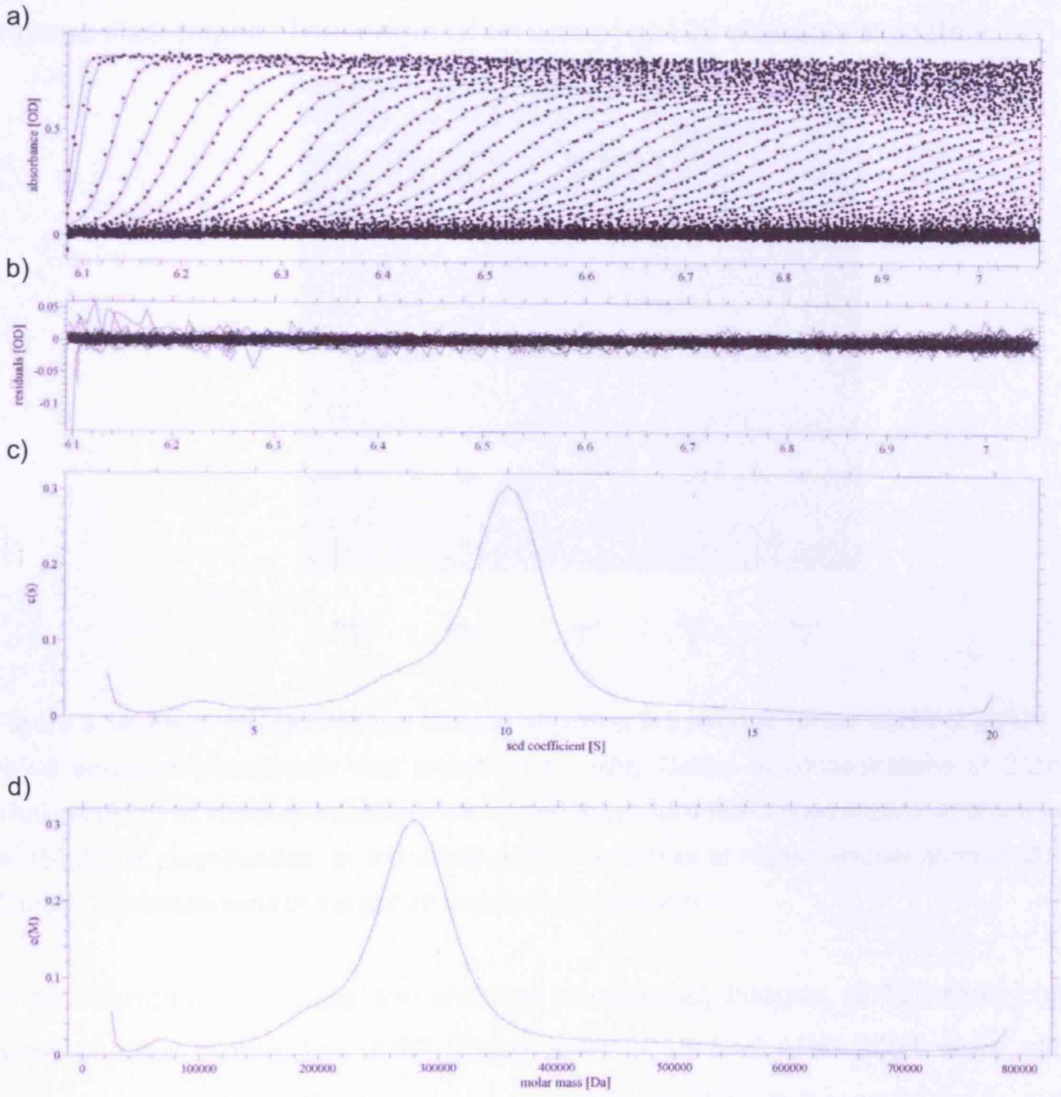


Figure 3.33: Estimation of gp120 molecular mass from sedimentation coefficient

This figure shows the raw data (**a**) together with the residuals (**b**) and the calculated sedimentation coefficient (**c**) and the estimated molecular weight distribution(**d**). Analyses were performed using SEDFIT (Schuck, 2000).

3.3.4.4 Transmission Electron Microscopy

Electron microscopy was used to determine the shape and size of the HIV-2 gp120 envelope glycoprotein (Figure 3.34). Species present contained 3 or 4 'arms' which are probably dependent on the orientation being viewed and 'flattening' effects due to adsorption onto the carbon-coated copper grids. This is compatible with a trimeric state with three arms representing the gp105 heads and the other the gp41 trimeric stalk region. The length of an arm of gp120 was approximately 14-18nm.

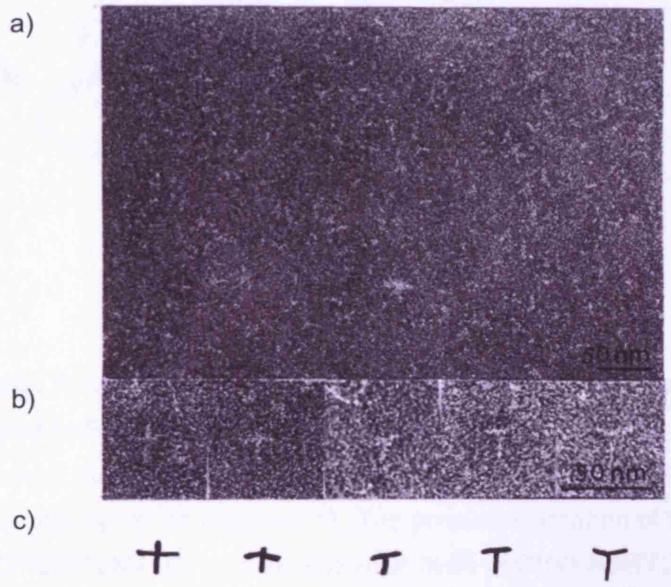


Figure 3.34: Electron Microscopy Images showing the various forms of HIV-2 gp120
HIV-2 envelope glycoprotein was provided to Lesley Calder at concentrations of 0.2mg/ml and dilutions (PBS) of this purified protein were used to produce the images shown. **a:** broad field image at 150,000 x magnification. **b:** individual gp120 molecules at higher magnification (x 200,000). **c:** Cartoon representations of the gp120 molecule present in b.

In an attempt to 'orientate' the electron microscopy images, gp120-MAb complexes were studied. Antibodies ARP 3083, ARP 3085 and ARP 3091 were allowed to form complexes with the envelope glycoprotein. The epitopes of these antibodies are known (C1, V2 and V3 respectively), and if it is assumed that the HIV-1 (gp120) and HIV-2 (gp105) will have similar structures based on conservation of important amino acid residues (notably cysteines), the appointed locations of the epitopes can be indicated on the HIV-1 gp120 structure (Figure 3.35). However as has been shown for interaction of influenza HA with MAbs (Wrigley et al., 1983) a variety of complexes (e.g. 1HA:1MAb, 2HA:1MAb, 1HA:2MAB, 2HA:2MAb, etc)

can form. This was shown to be the case for HIV-2 gp120:MAb complex formation (Figure 3.36).

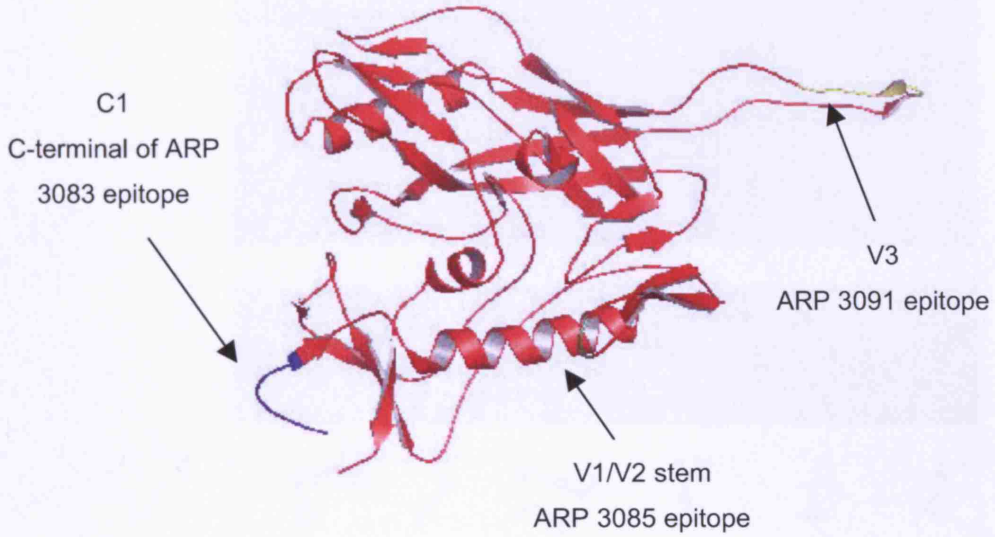
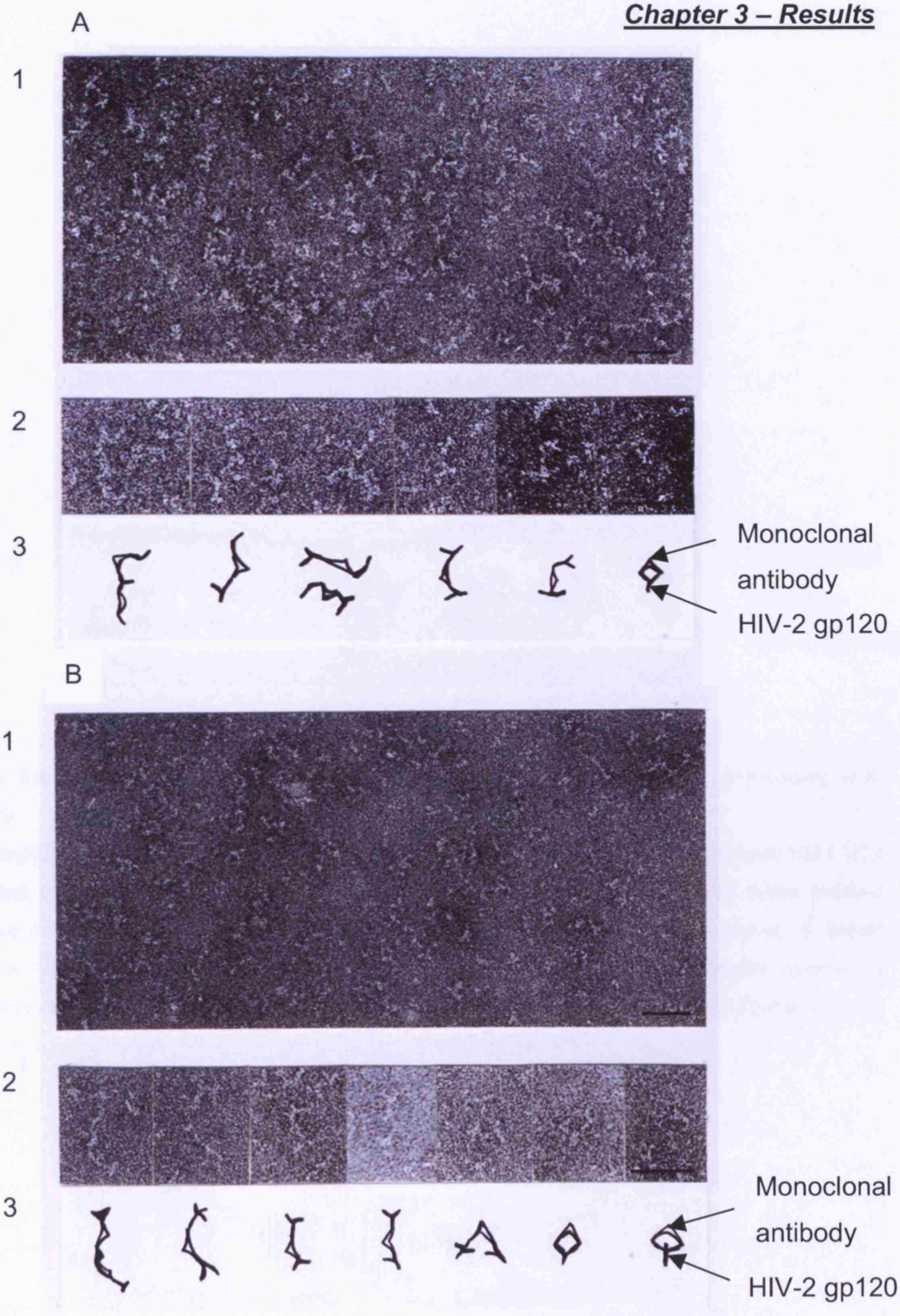


Figure 3.35: Crystal Structure of HIV-1 gp120 indicating the probable location of epitopes of the antibodies used in Electron Microscopy with HIV-2 gp120.

The crystal structure of the V3 loop containing HIV-1 gp120 (PDB No. 2B4C) (Huang et al., 2005) is shown in the same orientation as Figure 1.11. The presumed location of the epitopes are indicated for ARP 3083 (DDYQE**ITLNVTE**) in blue, and ARP 3091 (**SGHVFHSHYQ**) in yellow. However, the epitope for ARP 3085 (GEEETINCQ) is not present on this structure as the V1/V2 loop has been removed, therefore the stem of that loop has been indicated, shown in green.


A
1
2
3
Monoclonal
antibody
HIV-2 gp120
B
1
2
3
Monoclonal
antibody
HIV-2 gp120

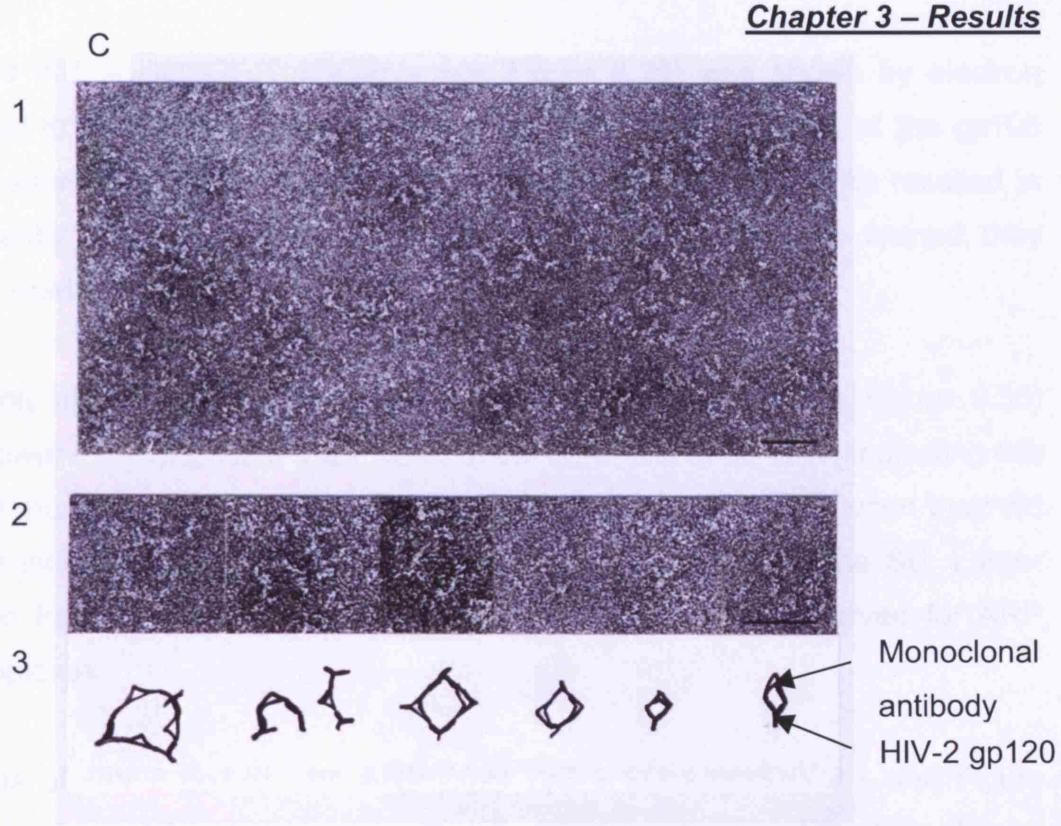


Figure 3.36: Electron Microscopy Images showing complexes of Monoclonal antibodies with HIV-2 gp120

HIV-2 envelope glycoprotein and monoclonal antibodies (ARP 3083 (A), 3085 (B) and 3091 (C)) were provided to Lesley Calder at concentrations of 0.2mg/ml. Dilutions (PBS) of these purified proteins were mixed to allow complex formation and used to produce the images shown. 1: broad field image at 150,000 x magnification. 2: individual HIV-2 gp120-monoclonal antibody complexes at higher magnification (x 200,000). 3: Cartoon representations of the HIV-2 gp120-monoclonal antibody complexes present in 2. The scale bar represents 50nm.

ARP 3083 (C1 – DDYQEITLNVTE – see Figure 3.35) was shown by electron microscopy to bind to two distinct lobes of gp120, inside the bowl of the gp105 heads at approximately 90° to the axis of gp120 (Figure 3.36A). This resulted in predominantly 1:1 and 2:1 complexes. When larger complexes were formed, they tended to yield 'zig-zag' strings.

Complexing of HIV-2 with ARP 3085 (V2 – GEEETINCQ – see Figure 3.35) showed fewer 1:1 complexes than either of the other two antibodies, indicating this may be a more difficult complex to form (Figure 3.36B). However, when they did form they indicated that the antibody was binding to the tips of the SU. Larger complexes formed longer more extended strings than those observed for ARP 3083 complexes.

Complexes of HIV-2 gp120 with ARP 3091 (V3 – SGHVFHSHYQ – see Figure 3.35) again showed that the binding site was very near the tip of the lobe (Figure 3.36C). When larger complexes (2:2 and 3:3) form ring structures, suggesting that the binding angle was such that the antibody is tilted towards the middle of the gp120. Overall, the binding pattern of the 3 MAbs support HIV-1 gp120 and HIV-2 gp105 having similar structures as inferred in Figure 3.35.

3.3.5 Conclusion

The purification protocol developed for HIV-2_{ROD} rgp120 construct 39 yielded a product of approximately 95% purity (section 3.3.2.3) that was shown by iso-electric focusing to have a weakly acidic pI of between 3.8 and 5.2 (section 3.3.2.4).

Functionality of the HIV-2_{ROD} envelope glycoprotein was shown through its ability to bind ARP 3087, 3088 and 3089 (antibodies known to bind to conformation-dependant epitopes) (McKnight et al., 1996)under conditions of native western blot (See Appendix Table 2). Additionally, ELISA based methods showed that the purified protein bound CD4 although this was more difficult to confirm using BIAcore methodology (discussed later in section 4.4).

Secondary structure analyses, using far UV circular dichroism and fluorescence spectroscopy indicated the presence of a folded, correctly proportioned (in terms of α-helices and β-sheets – Table 4.1), protein. In terms of tertiary structure, the HIV-2_{ROD} envelope glycoprotein was shown to be an oligomer by the molecular weights estimated using native PAGE, size exclusion chromatography and dynamic light scattering. However, molecular weight determinations by these techniques are affected by the shape of the molecule. For extended glycoproteins, like HIV-2_{ROD} gp120, artificially high molecular weight estimates result. Therefore, more accurate methods of analysis were used, analytical ultracentrifugation and transmission electron microscopy, to show that the HIV-2_{ROD} envelope glycoprotein was a trimer.

This extensive characterisation of the HIV-2_{ROD} envelope protein indicated that it was a correctly folded trimeric protein suitable for crystallisation trials.

3.4 Crystallisation Trials

3.4.1 Aim

Using the HIV-2_{ROD} rgp120 construct 39 purified product, the aim of this study was to establish chemical conditions that would yield crystal of a suitable size and quality to generate high resolution diffraction patterns, allowing determination of the rgp120's molecular structure

Having established that the 39.5 8 gp120 was functional with respect to binding of CD4 and conformation-specific MAbs, showed evidence of folding compatible with other reports in the literature, had an oligomeric state consistent with trimer formation and was of a high purity, crystallisation trials have been initiated.

3.4.2 Manual Setup

A manual PS screen (Majeed et al., 2003) was set up in 24 well plates using 0.25µl aliquots of a purified gp120 preparation at 1.3mg/ml. Several conditions which utilised Polyethylene glycols (PEGs) and organic solvents produced various precipitates. Examples of these are shown in Figure 3.37.

Four conditions were chosen to optimise further based on their abilities to produce medium to heavy precipitates in the presence of a variety of organic components (Table 3.5 and Appendix Table 3 - Reagents 27, 41, 48 and 49). To optimise, the mother liquor was diluted so that the principal reagent reduced by 5% in each step (Table 3.5).

Mother Liquor	PEG	Organic	pH	Additive
27	25% PEG 1500	*30% MPD*	4.5	
41	20% PEG 3350	*25% PEG 400*	8.5	0.1M $MgCl_2$
48	*25% PEG 3350*	15% MPD	6.5	0.2M $LiSO_4$
49	*25% PEG 3350*	4% Isopropanol	7.5	0.1M $CaCl_2$

Table 3.5: Conditions optimised in gp120 crystallisation trials

Mother liquors 27, 41, 48 and 49 from the PS Screen (Majeed et al., 2003) were diluted to optimise conditions by 5% at each step of the principal reagent (highlighted in Italics). MPD = (±) -2-Methyl-2,4-pentanediol, PEG = Polyethylene Glycol.

This tray produced spherulites in two wells corresponding to conditions 41 with 10% PEG 400 and 49 with 10% PEG 3350. Figure 3.38 shows the condition 49 result. Further screens set up around both these conditions with altered pH and concentrations of the principal reagent are underway.

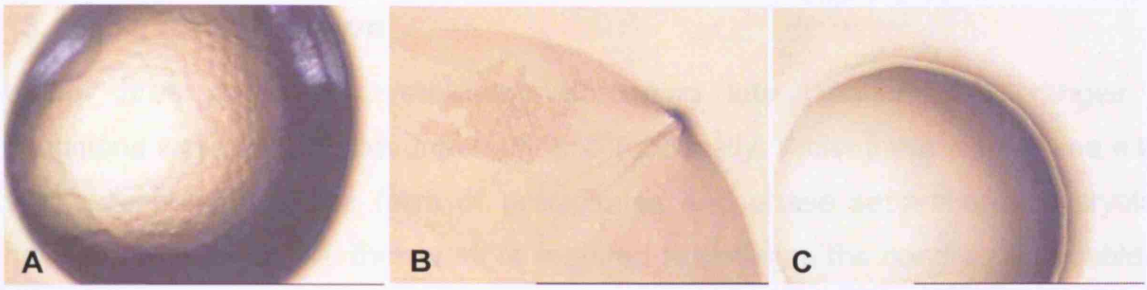


Figure 3.36: Typical Responses in gp120 crystallisation trial drops

HIV-2 envelope glycoprotein at a concentration of 1.3mg/ml was used to set up these drops, 325ng/drop was used. The PS Screen system was used (Majeed et al., 2003) and representative results for three of the conditions are shown. **A:** shows a typical phase separation response (PS Screen – 24). **B:** shows a typical site specific precipitation (PS Screen – 29). **C:** shows a clear drop (PS Screen – 22). All drops were photographed 3 months following set-up.

Figure 3.37: Optimised gp120 crystallisation condition trial.

HIV-2 envelope glycoprotein at a concentration of 1.3mg/ml was used to set up these drops, 325ng/drop was used. PS Screen No 49 (Majeed et al., 2003) produced a medium to heavy regular precipitate (**A**), the 10% PEG 3350 dilution of this mother liquor produced drops containing spherulites (**B**), which then further condensed (**C**). Photographs were taken 91 (**A**), 74 (**B**) and 160 (**C**) days post set up of the trials.

3.4.3 Robotic Setup

Two 96 well plates were set up using The Classics Suite (Nextal Biotechnologies), Stura Footprint Screens MDI-20 (Molecular Dimensions Ltd) and Macrosol™ MDI-22 (Molecular Dimensions Ltd) collections of mother liquors. Results comparable to those shown in Figure 3.38A and B were obtained for approximately 8.3% (The Classics Suite) and 66.7% (Stura/Macrosol Kits) of the wells 3 months following set up of the strips (see Appendix Table 4). Further crystallisation trials are ongoing in attempts to generate crystals suitable for X-ray crystallography.

3.4.4 Conclusion

Using three different crystallisation-screening kits (section 3.4), ranges of conditions were set up both manually and robotically. Whist there were some early visible responses in the form of precipitates and phase separation, no crystals have formed as yet. Further work is required to optimise the conditions suitable to establish crystals that may involve alterations to the chemical conditions and/or the rgp120 itself (e.g. deglycosylation and or co-crystallisation with ligands such as MAb Fab fragments and/or CD4; discussed in section 4.6).

Chapter 4

Discussion

4 Discussion

As discussed in the introduction the importance of HIV envelope glycoprotein in the viral life cycle and the pathogenesis of the disease in the host is clear. Env not only determines which cell type is infected but also it seems able to control the production of β-chemokines which are able to regulate infection. Thermodynamic studies indicate large changes in the structure of both the SU and TM glycoproteins on binding to the cellular receptor CD4 (Myszka et al., 2000). Further conformational changes are required for fusion to take place and this involves the interaction of the SU glycoprotein with a chemokine co-receptor (CCR5/CXCR4) allowing six-helix bundle formation of the ectodomain of the TM and fusion peptide insertion into the host cell membrane (Figure 1.18). The end points of these conformational changes have been shown by the crystal structures of the unliganded SIV core SU (Chen et al., 2005a; Chen et al., 2005b), the liganded HIV-1 core SU glycoproteins (Figure 1.13), where binding of CD4-induced antibodies may be equivalent to binding of chemokine co-receptor (Huang et al., 2005; Kwong et al., 2000a; Kwong et al., 1998), and portions of the HIV-1 gp41 ectodomain (Figure 1.14) (Weissenhorn et al., 1997b) supported by NMR structures of a similar portion of the SIV gp41 (Figure 1.11) (Caffrey et al., 1998). A thorough literature search has not revealed any information on the tertiary structure of the HIV-2 envelope glycoprotein.

4.1 Env-gene templates and Construct Generation

Both membrane proteins and proteins which are to be secreted require an N-terminal hydrophobic signal sequences which targets the protein to the endoplasmic reticulum (Walter, Gilmore, and Blobel, 1984). Experiments in various cell lines have demonstrated that the endogenous HIV envelope glycoprotein signal sequence retards the folding of the protein (Li et al., 1996; Li et al., 1994). Also, during the early stages of biosynthesis there is slow and inefficient cleavage of the signal sequence from HIV Env (Li et al., 2000). Therefore, various signal sequences have been investigated including Erythropoietin signal peptide (Herrera et al., 2000) and tissue Plasminogen Activator (tPA) signal peptide (Chapman et al., 1991; Golden et al., 1998). When compared, equivalent amounts of HIV-1

gp120 were expressed when either of these were used (Herrera et al., 2000). Our system utilised a tPA signal peptide under control of a Cytomegalovirus promoter.

Fibritin is a homotrimer, a 52KDa product of the gene *wac* (whisker antigen control) derived from the bacteriophage T4. The crystal structure of fibritin has been solved (Strelkov et al., 1996; Strelkov et al., 1998). This showed that the C-terminal domain of the protein initiated and stabilised the formation of a coiled coil and hence the trimer (Tao et al., 1997). The C-terminal domain of this protein, residues 456-483 (QQEKNMYELQKGYIPEAPRDGQAYVRKGEWVLLSTFL) was utilised successfully by Yang and colleagues (Yang et al., 2002) to stabilise HIV-1 envelope glycoprotein trimers. Hence, we utilised this region in some of our constructs to stabilise trimers formed (see section 3.3.3.4).

Initially, detecting expression of the four different gp120's derived from patient samples constructs proved difficult. The only samples which appeared to contain HIV-2 gp120 were cell lysates. This was obviously a barrier to further work as secreted protein was required. All these constructs were terminated at the proposed start of the gp36 membrane anchor domain. Close inspection of the constructs translation products revealed a C-terminal 21-amino acid sequence that was highly hydrophobic, more so than equivalent HIV-1 constructs being expressed in the laboratory. It was considered that this sequence may have caused retention of HIV-2 gp120 in cell membranes. Indeed, it has been observed for HIV-1 gp140 constructs that truncation at the 2F5 epitope (Muster et al., 1993) results in better secretion of gp140 ((Jeffs et al., 2004) and Daniels et al., unpublished). The design of the HIV-2_{ROD} constructs included this information, which therefore extended the number of constructs to eight (with and without (i) cleavage site mutations, (ii) the fibritin domain and (iii) the 21 hydrophobic amino acid residues on the C-terminal).

Interestingly, *E.coli* transformed with the construct gp120FIB L (Table 2.2) became flattened and non-viable following 36-48h incubation suggestive of cell lysis, whereas transformants were stable for gp120FIB cs L and gp120FIB S constructs. It has been suggested that the membrane proximal region of HIV-1 gp41, which

contains several tryptophans three of which are conserved throughout HIV-1, HIV-2 and SIV, may become sequestered in the membrane during fusion (Salzwedel, West, and Hunter, 1999). This region which has α-helical properties has also been implicated in the destabilisation of membranes during cell fusion (Nieva et al., 1994; Rafalski, Lear, and DeGrado, 1990; Slepushkin et al., 1990; Suarez et al., 2000). It is possible that the *E.coli* expressed some HIV-2 Env protein which, in the context of construct gp120FIB L, was able to cause membrane destabilisation of the *E.coli*.

Several groups have mutated the cleavage site between gp120 and gp41 in order to produce the full length ectodomain of HIV-1 envelope glycoprotein (Binley et al., 2002; Chakrabarti et al., 2002; Yang et al., 2000a). We utilised this technique in order to make full length ectodomains of HIV-2 envelope glycoprotein. It proved difficult to detect HIV-2 gp36 (construct 1) as the reagents we were able to access were poor. Therefore, the majority of the experiments undertaken in this PhD have been with the stable cell line expressing construct 39 (Table 2.1 – a cleavage mutant, fibritin domain containing construct of HIV-2_{ROD} gp120).

4.2 Screening for HIV-2 gp120 expression

HIV-2 envelope glycoprotein constructs have been produced for expression in *E. coli* (Ulrich, Siakkou, and Kruger, 1992; Zuber et al., 1990), baculovirus/Insect cells (Kang, 1997), vaccinia virus/mammalian cells (Mulligan et al., 1992a; Otteken, Voss, and Hunsmann, 1993) and mammalian (CHO *lec* 3.2.8.1) cells (Sourial et al., 2005).

It has been shown that HIV envelope glycoprotein produced in the absence of glycosylation, either by deletion of the signal sequence of HIV-1 gp120 (Ellerbrok et al., 1992) or by synthesis in the presence of tunicamycin (Murphy et al., 1990), failed to bind CD4. This function is however present in glycosylated HIV-1 gp120 which has the carbohydrate structures removed by endoglycosidase H or an endoglycosidase F/N glycanase digestion (Bahraoui et al., 1992). Therefore, the N-linked glycosylation of HIV envelope glycoprotein is essential for production of a functional CD4 binding site (Li et al., 1993). The type of glycosylation has been

shown to be dependant on host cell-specific factors rather than on the amino acid sequence of the HIV-2 Env (Liedtke et al., 1994; Liedtke, Geyer, and Geyer, 1997). Studies have shown that baculovirus-derived HIV-1 envelope glycoprotein has a weaker affinity for CD4 and monoclonal antibodies than CHO derived HIV-1 envelope glycoprotein (Moore et al., 1990a).

It has been reported that the altered glycosylation patterns produced by mutant CHO cell lines such as Lec23 (lacks α-glucosidase I activity), Lec 10 (has increased GlcNAc transferase III activity), Lec 1 (lacks GlcNAc transferase I) and Lec 3.2.8.1 (lacks GlcNAc transferase activity and has decreased activity of CMP – NeuNAc and UDP – Gal translocases) does not affect the quantity of Env produced (Fenouillet, Miquelis, and Drillien, 1996). More importantly, the glycosylation modified forms of Env retained immunogenicity of the V3 loop, processing of gp160, transport to the cell surface and associated membrane fusion activity with $CD4^+$ cells and secreted Env still bound CD4. Therefore, it is possible to produce HIV envelope with a more homogeneous population of attached carbohydrate structures. However, in our hands CHO *lec* 3.2.8.1 (Stanley, 1989) cells produced a lower yield of secreted Env in comparison to CHO K1 produced HIV-1 gp140 (Daniels & Hickling unpublished results). Therefore CHO K1 cells were used to produce stable cell lines for this HIV-2 Env-based PhD study.

Foreign gene expression in CHO cells is usually achieved through use of a CMV promoter and expressing cell-lines are generated by use of selection systems based on either dihydrofolate reductase (DHFR), using methotrexate (MTX) resistance, or glutamine synthetase (GS), using methionine sulfoxamine (MSX) resistance. The plasmid used during the course of this PhD (pEE14tPA2DCD4, Figure 2.2) utilised the glutamine synthetase system. The GS enzyme catalyses the production of glutamine from glutamate and ammonia. MSX binds to the GS enzyme and prevents the production of glutamine. Gene amplification occurs when the CHO cells are exposed to increasing concentrations of MSX.

A range of transfection kits (Section 2.2.2) were assessed. It was found that effectene (Qiagen) was the most successful for transfection of both 293T and CHO K1 cells, whilst Lipofectamine (Invitrogen) was best for CV-1 cells.

Sodium butyrate has been shown to increase the productivity of IgG from CHO K1 without altering the glycosylation or biological activity (Mimura et al., 2001). Therefore, 2mM sodium butyrate was used in the last stages of protein production to increase the yield of HIV-2 gp120. The use of serum-free suspension cell culture, requiring adaptation of cell lines, was considered but not employed as we (Billington et al., unpublished) and others (Jeffs et al., 2006; Rosser et al., 2005) have found lower yields of HIV-1 Env constructs compared to adherent cell culture of CHO K1.

Towards the end of the current PhD studies, immunofluorescence experiments showed that approximately 60% of the cells in the stable cell line were expressing HIV-2_{ROD} gp120. Protein yields might be improved by either subjecting the cell-line to another round of MSX selection or subdividing the cell line by limit dilution, to generate higher percentages of cells expressing HIV-2_{ROD} gp120.

4.3 HIV-2_{ROD} gp120 Capture Systems

Purification of HIV-2 envelope glycoproteins has been carried out by two groups. Zhang and colleagues (Zhang et al., 2001b; Zhang et al., 2002) produced HIV-2 SU in the methyltropic yeast *Pichia Pastoris* and purified the expressed protein (56.4mg/l) using a Sephadex-G 100 column. Sourial and colleagues (Sourial et al., 2005) expressed their truncated and V1/V2 deleted HIV-2 gp125 constructs in CHO *lec* 3.2.8.1 cells and purified them (2mg/l) by a single step strategy using *Galanthus nivalis* lectin.

Several HIV-2 gp120 purification strategies were attempted. Purification using the C-terminal hexa-His tag was not successful. It would appear that the six histidines were unavailable for binding and this was supported by negative western blot results when probed with an anti-his tag antibody. Ion exchange chromatography provided little improvement in the purity of the HIV-2 gp120 TCSN (Figure 3.9).

This is not surprising due to the heterogeneous charge of the HIV-2 gp120 produced, presumably relating to the heterogeneity of carbohydrate moieties attached to the protein, as is shown by the large smear on an isolelectric focusing gel corresponding to a pI of pH 3.8 – 5.2 (Figure 3.26).

It has been known for several years that HIV envelope glycoproteins bind to mannose binding lectins (Gilljam, 1993; Mahmood and Hay, 1992; Shibuya et al., 1988). Lectins from the *Amaryllidaceae* family have even been suggested as microbicides to prevent HIV infection (Balzarini et al., 2004). Several groups (Jeffs et al., 2004; Sourial et al., 2005) have successfully used lectins especially *Galanthus nivalis* lectin to purify HIV envelope glycoprotein produced in CHO cells. The efficacy of four mannose binding lectins for HIV-2 gp120 purification was investigated. PSA had minimal binding affinity for HIV-2 gp120 (Figure 3.10) whereas Con A had strong affinity for construct 39.5 8 as shown by the need to incubate at 50°C to achieve HIV-2 gp120 elution. Although, construct 1.2 4 bound Con A, the positive elutions showed very little purification (Figure 3.12). GNA and HHL bound the gp120 components of HIV-2_{ROD} constructs 1 and 39, but had very weak affinity for the SU (gp105) portion of construct 1. This suggests that, in the CHO K1 system, carbohydrates on the gp36 components are the major determinants of GNA and HHL binding. If this is so,

(i) use of lectins with specificity for SH containing carbohydrates may be warranted as there is evidence for the gp41 carbohydrates of HIV-1 being sulphated (Bernstein and Compans, 1992; Shilatifard et al., 1993).

(ii) the presence of the gp36 component may affect the glycosylation of the SU (gp105) component as GNA has been used to purify HIV-1 gp120 and gp140 constructs produced in CHO K1 cells (Gilljam, 1993; Hinkula et al., 1994; Jeffs et al., 2004). In this context, the work of Sourial *et al.,* used CHO *lec* 3.2.8.1 with different glycosylation properties to CHO K1 (Sourial et al., 2005).

Overall, as an initial step, GNA yielded better purification of construct 39 HIV-2_{ROD} gp120 from TCSN than did HHL (Figure 3.15). However, the purity was not adequate for crystallisation trials so further steps were required (Figure 3.15c).

Current HIV/SIV Env SU crystal structures have been from protein which has been purified using an immunoaffinity column (Chen et al., 2005a; Kwong et al., 1998) followed by size exclusion (Superdex 200) chromatography. Hence, we considered that the appropriate antibody might be able to yield a less heterogeneous population of HIV-2 gp120. We obtained eleven hybridoma cell lines from CFAR (NIBSC, UK) with the kind permissions of Dr Q Sattentau and Dr A McKnight. All eleven produced monoclonal antibodies raised against baculovirus expressed HIV-2 gp105 (McKnight et al., 1996; Sattentau et al., 1993). To determine the best candidate for an immunoaffinity column, binding of each monoclonal antibody to partially purified HIV-2_{ROD} construct 39 was assessed. Immunoprecipitation, native gel/western blots, surface plasmon resonance BIAcore and vivaspin column assays were performed for each antibody (Table 3.3 and Appendix Table 2). The experiments undertaken indicated that ARP 3085 (V2 – GEEETINCQ) would be a good candidate for an immunoaffinity column. Hence, production of this antibody was scaled up to allow production of a Cyanogen Bromide coupled immunoaffinity column. The conditions of immunoaffinity purification were optimised to allow production of gp120 that could be further fractionated using size exclusion chromatography. These consisted of a post-binding, pH 5.5 wash (which removed contaminants) followed by a low pH (pH 2.5) elute.

Whilst existing HIV/SIV SU structures have been obtained with protein that had been subjected to Superdex 200 gel filtration (separation range 10,000–600,000Da), the HIV-2_{ROD} gp120 was shown not to interact with the Superdex 200 column (Figure 3.19) as it was eluted in the void volume (K_{av} = <0) such that no separation of the loaded proteins occurred. The HIV-2_{ROD} gp120 protein did interact with the beads (K_{av} = 0.3) in the Superose 6 column (separation range 5 x 10^3 – 5 x 10^6Da: Figure 3.19 and 3.20) allowing some separation of the contaminants within the sample. However, an ideal column for gel filtration will elute the specified protein with a K_{av} of 0.5. This might have been achieved for HIV-2_{ROD} gp120 with a Sephacryl S 500 HR column (Separation range 4 x 10^4 – 2 x 10^7Da) and is an avenue to explore with further work.

DLS was performed on the Superose 6 HIV-2_{ROD} gp120 peak fractions and this yielded a polydispersity of ~23% and an estimated molecular weight of ~580KDa. This level of polydispersity is acceptable for crystallisation trials (D'Arcy, 1994; Ferre-D'Amare and Burley, 1997), but the DLS estimated molecular weight is high. The latter estimate is based on the hydrodynamic radius and this may have been 'over-estimated' as it is likely that HIV-2 gp120 forms a large trimeric protein (Figure 3.34), the hydrodynamic radius of which may be increased by the mobility of attached carbohydrate complexes.

In the course of the established purification process (GNA lectin chromatography, Immunoaffinity Chromatography, Superose 6 size exclusion, DLS selection of Superose 6 fractions) approximately 80% of HIV-2 gp120 was lost (typically 275µg recovered from 1.3mg starting material), with the bulk of this loss occurring during the immunoaffinity chromatography step. However, the final purified gp120 possessed properties that made it suitable for crystallisation trials and warranted further characterisation.

4.4 HIV-2_{ROD} gp120 characterisation

Binding studies indicated that the HIV-2_{ROD} gp120 product was functional. In an ELISA format it was shown to bind CD4 and conformation-dependant antibodies were shown to bind it by native gel/western blot and BIAcore assays. Unfortunately, despite numerous attempts using different conditions ((i) binding CD4 to the CM5 chip and passing gp120 over and vice versa, (ii) using a range of protein concentrations, and (iii) using a variety of buffer systems,) a reproducible reading for CD4 binding affinity (K_D) was not achieved using the BIAcore. Reports have suggested that HIV-2 gp105's affinity for soluble CD4 is 10 to 25 times less (4.5×10^{-8} - 7×10^{-8}M) than that reported for HIV-1 (Bahraoui et al., 1992; Moore, 1990). However, more recently studies have shown trimeric HIV-1 gp140 to have a weaker binding affinity for CD4 (µM) than monomeric HIV-1 gp140 (nM) (Jeffs et al., 2004; Srivastava et al., 2002; Staropoli et al., 2000; Yang et al., 2000a), which could possibly be due to the stoichiometry of the reaction. Hence, this could have implications for the binding of trimeric HIV-2 gp120, not least in the increased amount of material required to achieve an accurate K_D reading using SPR BIAcore.

The isoelectric points predicted from the gp120 sequence by computer programs were weakly basic with values of 7.937 (Sednterp) and 7.77 (ProtParam). However, experimental data produced during the course of this thesis have shown the pI of this protein to be acidic with values of ~pH 6 (IEX) and a range between pH 3.8-5.2 shown by Isoelectric focusing (Figure 3.26). This concurs with results from Zhang and colleagues who have shown that their HIV-2 gp105 produced in yeast has a pI of 5.2 (Zhang et al., 2002). Also, studies within our laboratory have shown that the HIV-1 gp140 has a pI in the range of 4-5 (Billington et al., unpublished). The acidic nature of this protein is due to the high numbers of sialic acid residues present within the carbohydrates attached to the protein.

Although secondary structure prediction programs and methods of measurement such as CD and Fourier transform infrared spectroscopy (FTIR) are likely to contain errors as high as ±5% for the % of α-helix and β-sheet calculated/measured, prediction was used to determine the secondary structure of a range of HIV/SIV glycoprotein constructs (Table 4.1). Sequences were submitted to www.PredictProtein.org which used various programs to predict secondary structure. PHDsec (Rost and Sander, 1993) and PHDhtm (Rost, Fariselli, and Casadio, 1996) versions 1.96 software used multiple sequence alignments to predict 31% α-helix and 28% β-strand and 42% neither helix nor strand for HIV-2_{ROD} gp120 ectodomain (Table 4.1[b]). A similar result was obtained for HIV-1_{NL43} ectodomain (Table 4.1[c]), supporting the suggestion that HIV-1/HIV-2 glycoproteins have similar structures (Douglas, Munro, and Daniels, 1997). Such similarity also held for HIV-1_{NL43} (Table 4.1[g]) and HIV-2_{ROD} (Table 4.1[h]) SU domains, though the ratio of α-helix:β-sheet was significantly decreased. This probably reflects the predominantly α-helical structure of the ectodomain of gp41/36 (Caffrey et al., 1998; Weissenhorn et al., 1997b). Interestingly secondary structure values predicted for HIV-1_{JRFL} and $SIV_{MAC\ 32H}$ (Table 4.1[d + e]: figures in parenthesis) agreed well with their known X-ray crystallisation structure (Chen et al., 2005a; Chen et al., 2005b; Huang et al., 2005), despite HIV-1_{JRFL} only being modelled on its known structure (PDB No. 2B4C). This again demonstrates the value of secondary structure prediction and indicates a lack of secondary structure modification due to

ligands (CD4 and X5 MAb) being bound and carbohydrates being removed in the case of HIV-1_{JRFL} (Table 4.1[d]).

The predicted values of secondary structure acted as guidelines for subsequent CD which was used to determine if our HIV-2_{ROD} gp120 (construct 39) was folded and if so its secondary structure content. CD also allowed the determination of the stability of the protein at different temperatures, pH's and with denaturants. Due to the limited amount of pure HIV-2 gp120 (construct 39) available, far-UV CD was used to determine the secondary structure content which was determined to be 22% α-helix, 24% β-sheet, 23% turn and 29% unstructured (Table 4.1[a]), a small percentage (approx 2%) of the β-sheet content could be due to the C-terminal domain of the fibritin (Figure 1.15). Far-UV CD also indicated that the HIV-2_{ROD} gp120 glycoprotein was thermally stable up to at least 85ºC. This is not unusual for large proteins (Dr S. Martin, Physical Biochemistry, NIMR, personnel communication). Other groups (Chen et al., 2000; Sourial et al., 2005) have used CD to investigate the secondary structure of various HIV envelope glycoprotein constructs and stated that they are comparable with the results of Hansen for HIV-1 gp120 (Hansen et al., 1996). Prediction via their COMBI method gave values of 20.2% α-helix and 33.3% β-strand.

Given the % error for secondary structure prediction and methods of measurement (see above), the values for similar constructs, SU (Table 4.1[d-h], including the FTIR measurement for HIV-1_{BH10}) and ectodomain (Table 4.1[b,c]) are comparable. However, whilst for HIV-2_{ROD} gp120 (construct 39.5 8: Table 4.1[a]) there is correlation of the CD-determined β-sheet content with that predicted for HIV-2_{ROD} and HIV-1_{NL43} ectodomain, the level of α-helix (22%) is considerably less than the predicted values (31/32%) (Table 4.1[b,c]). This level of difference is at the limits of experimental error and possibly results from our gp120 being a trimer (Figure 3.34) whilst prediction is based on a monomeric state. Alternatively the trimeric state, which may have been stabilised partially by the removal of the gp105/gp36 processing site, may represent a pre-fusion, non-energy minimised conformation.

Virus	Construct[1]	Expression	Technique	% α-helix	% β-sheet	Reference
HIV-2[a]	gp105 and ectodomain gp36 (ROD)	CHO K1	Far-UV Circular Dichroism	22	24	This Thesis
HIV-2[b]	gp105 and ectodomain gp36 (ROD)	N/A	PHDsec PHDhtm Prediction Software	31	28	(Rost, Fariselli, and Casadio, 1996; Rost and Sander, 1993)
HIV-1[c]	gp120 and ectodomain gp41 (NL43)	N/A	PHDsec PHDhtm Prediction Software	32	28	(Rost, Fariselli, and Casadio, 1996; Rost and Sander, 1993)
HIV-1[d]	gp120 ΔV1/V2 and N and C termini (bound CD4 and X5-Fab) (JR-FL)	Drosophila	X-ray Crystallography	13.3 (16)[2]	33.3 (35)[2]	(Huang et al., 2005)
SIV[e]	gp120 ΔV1/V2/V3 and N and C termini (MAC 32H)	Drosophila	X-ray Crystallography	16.2 (16)[2]	36.8 (34)[2]	(Chen et al., 2005a; Chen et al., 2005b)
HIV-1[f]	gp120 (BH10)	vaccinia virus in CV1	Fourier transform infrared spectroscopy	22	38	(Decroly et al., 1993)
HIV-1[g]	gp120 (NL43)	N/A	PHDsec PHDhtm Prediction Software	16	38	(Rost, Fariselli, and Casadio, 1996; Rost and Sander, 1993)
HIV-2[h]	gp105 (ROD)	N/A	PHDsec PHDhtm Prediction Software	17	34	(Rost, Fariselli, and Casadio, 1996; Rost and Sander, 1993)

Table 4.1: Analysis of HIV Env secondary structure data

[1] The strain analysed is shown in parenthesis. [2] The results of secondary structure prediction are shown in parenthesis (www.PredictProtein.org (Rost, Fariselli, and Casadio, 1996; Rost and Sander, 1993)). No HIV/SIV-related structures were recruited to alignments for secondary structure prediction for HIV-2[b], HIV-1[c], HIV-1[g] and HIV-2[h]. For HIV-1[d] the corresponding PDB file (2B4C) was recruited, whilst for SIV[e] the corresponding PDB file (2BF1) was not recruited while a series of HIV-

1 related PDB files (2B4C, 1G9M, 1RZJ, 1G9N, 1RZK, 1YYL, 1YYM and 1GC1) were. [a-h] To aid explanation in the text.

An indication that our HIV-2 gp120 was correctly folded came from fluorescence spectroscopy (Figure 3.28). An emission maximum of 345nm, compared to 356nm for free tryptophan, suggested that most of gp120 tryptophans were buried and therefore the protein was folded.

Analytical ultracentrifugation allows direct measurement of molecular weight of solutes in their native state and as they exist in solution, without having to rely on calibration and without having to make assumptions concerning shape. The method of sedimentation equilibrium analysis is firmly based in thermodynamics, therefore all the terms in the equations describing sedimentation behaviour are experimentally determinable. Hence, it is the most versatile, rigorous and accurate means for determining the molecular weight, hydrodynamic and thermodynamic properties of a protein.

Sedimentation velocity experiments allow the rapid and rigorous quantitative assessment of sample heterogeneity. Sedimentation and diffusion coefficients contain information concerning the size and shape of macromolecules and the interactions between them. Because the sample is examined in free solution and in a defined solvent, sedimentation methods allow analysis of purity, integrity of native structure and degree of aggregation, uncomplicated by interactions of the macromolecules with gel matrix or support. Notably, glycoproteins are known to show anomalous mobility in SDS acrylamide gels (Marciani and Papamatheakis, 1978).

Sedimentation equilibrium experiments provided data for a single species model and with the values stated above (Section 3.3.3.3.1) a molecular weight of 315.3KDa was determined. However, there were systematic deviations of the residuals between the data and fits suggesting a more complex situation. Therefore velocity analytical ultracentrifugation was undertaken and this showed that whilst there was some sample heterogeneity, the main peak yielded a

sedimentation coefficient of 10s which was converted to a molecular weight of 290KDa. The weight range (290KDa-315.3KDa) is consistent with the HIV-2_{ROD} gp120 oligomeric state being a trimer and comparable with current experimental data in the literature of molecular weights between 340KDa and 410KDa for various HIV-1 envelope ectodomain constructs (Chen et al., 2000; Zhang et al., 2001a).

Negative staining electron microscopy has been performed to assess the structures of soluble HIV-1 envelope glycoproteins (Center et al., 2004; Center et al., 2001; Pancera et al., 2005; Qiao et al., 2005; Schulke et al., 2002; Srivastava et al., 2003). However, a thorough literature search has not revealed any electron micrographs of soluble HIV-2 envelope glycoprotein. The HIV-2_{ROD} gp120 produced here was shown to exist as a three/four armed protein (dependent on the view presented), indicative of a trimeric protein (Figure 3.34), probably composed of a trimeric gp105/gp15 stalk and three gp105 heads. In an attempt to map/orientate these structures, gp120 was complexed with monoclonal antibodies of known specificity (Figure 3.36). Distinct binding patterns were observed for C1-, V2- and V3- specific MAbs. Most notably, complexes of ARP 3085 (V2- specific) indicate that whilst the V2 loop is located in the gp105 head, MAb binding is restricted in terms of 1:1 complexes and ring structures forming. This contrasts with ARP 3091 (V3-specific) which binds at the top of the gp105 heads and allows formation of a ring structure. These results show correlation with the known structure of HIV-1 gp120 (Huang et al., 2005; Kwong et al., 2000a; Kwong et al., 1998) especially with the known positions of the epitopes for ARP 3083 and ARP 3091 (Figure 3.35).

4.5 Crystal Trials

Although HIV-2_{ROD} gp120 characterisation suggested we have produced a protein suitable for crystallisation trials, to date such trials have been unsuccessful. Responses observed in the drops have varied from nothing, to precipitation of protein or phase separation (Figure 3.37). However, some leads have been followed up (Figure 3.38). Greater amounts of protein are required in order to be more thorough in refining the conditions identified in producing trials that yielded precipitates/phase separation. Such initial screen refinements should be carried out using the robotic system which utilises much smaller volumes of protein, followed by manual set up which may yield larger/better crystals.

4.6 Further Work

Consideration should be given to the production and purification of full-length HIV-2 gp140 constructs, as it has been shown that truncation of the cytoplasmic tail can alter the conformation of the SU of HIV-1/2 and SIV (Affranchino and Gonzalez, 2006; Edwards et al., 2002; Hoffman et al., 1999; Mulligan et al., 1992b; Spies et al., 1994). This would be an important avenue to follow, especially if considering these proteins for a vaccine study, but would out of necessity involve the use of detergents most of which are not compatible with crystallisation trials. Detergents such as LDAO and β-octyl glucoside have been used successfully in a limited number of cases (Ostermeier and Michel, 1997).

As well as looking to express the full-length HIV-2 gp140, shorter constructs than the soluble HIV-2 Env gp120 could be designed for structural studies. The truncated gp105 equivalent to that of the HIV-1 gp120 construct reported in 1998 could be designed (Kwong et al., 1998) and crystal trials attempted with/without sugars/ligands. Determination of the molecular structure of the HIV-2 soluble ectodomain of gp36 would not only complete the set (HIV-1 (Weissenhorn et al., 1997b)/ SIV (Caffrey et al., 1998)) but confirm that this part of the structure is similar/idenitical to that of the other primate lentiviruses. As mentioned previously, the V1/V2 loop of HIV-2 may be under a similar immune pressure (Almond et al., 1993; Damond et al., 2001; McKnight et al., 1996) to that of the V3 loop of HIV-1.

Hence, expressing a construct similar to that described by Huang et al., (Huang et al., 2005) but instead of maintaining the V3 loop retaining the V1/V2 loop may provide an interesting contrast. However, with all these constructs there is the problem of lack of reagents to assist in the detection and purification. This can be solved to some extent by using a cleavable tag that has reagents already raised against it such as a Haemagglutinin epitope placed on the N-terminus (Sourial et al., 2005).

Different expression systems should be trialled to assess whether they are more efficient than the current approach, examples of possible systems are the TriEx system from Novagen or the Flp-In™ T-Rex™ system from Invitrogen. These and other systems would allow production of protein in bacterial, baculovirus/insect cell and mammalian cell systems, though the bacterial system would likely only be useful for small fragments as glycosylation is known to be important for correct HIV-1 glycoprotein folding and function (Ellerbrok et al., 1992; Murphy et al., 1990). With the current system more stable cell lines should be set up expressing different constructs and possibly utilising the CHO *lec* 3.2.8.1 (Stanley, 1989) cell line which would produce protein with a homogenous population of sugars, although attempts with HIV-1 gp140 have been unsuccessful (Daniels et al., unpublished).

More recently a drug has come to our attention called Kifunensine which inhibits the mammalian Golgi enzyme, Mannosidase I, but does not appear to inhibit the α-mannosidase found in the ER. Kifunensine stops the production of complex chains allowing only $Man_9(GlcNAc)_2$ (Elbein, 1991) moieties to be added. This drug can be included in tissue culture, thereby reducing the heterogeneity of the carbohydrate structures on the expressed HIV-2 gp120 and allowing their easy removal with either peptide – N- glycosidase F (PNGase F) or endoglycosidase H (Endo H).

To further analyse HIV-2_{ROD} rgp120 secondary structure a series of proteolytic digests could be undertaken, followed by mass spectrometry analysis of the peptides produced to determine the disulphide bonding pattern within this Env protein. There are no reports of disulphide bonding patterns for HIV-2 Env other than the hypothesised bonding pattern suggested by Hoxie (Hoxie, 1991) which

was based on the extensive biochemical analysis carried out on the HIV-1 gp120 structure (Leonard et al., 1990). In addition, the glycosylation pattern of the expressed HIV-2 gp120 could be analysed in a similar way to Leonard's analysis of HIV-1's carbohydrate content (Leonard et al., 1990).

Raising of antibodies against the rgp120 antigen produced by the CHO K1 cell lines generated in the course of this PhD study should enhance the project. It may enable a two step purification process (Immunoaffinity followed by size exclusion chromatography) to be implemented, as has been achieved for HIV-1 gp140 studies (Billington *et al.,* submitted). A MAb for Immunoaffinity chromatography, directed against a different epitope in the protein to be purified, may provide significant enhancement of purified protein yields as this is the step where most HIV-2_{ROD} gp120 was lost during the three step procedure established here. Further, the antibodies produced may also improve detection of patient-derived gp120's compared to the reagents available to us in the course of this PhD study which were in the main raised against monomeric, baculovirus/insect cell-produced HIV-2_{ROD} gp105. Improved detection may lead to easier establishment of CHO cell lines expressing such constructs and assist in the purification of the HIV-2 gp120s produced. Provision of a wider range of gp120 constructs to carry into crystallisation trials, together with a wider range of monoclonal antibodies to act as co-crystallising agents (as Fabs), may improve chances of structure determination.

The electron microscopy results have provided the most interesting insights into the structure of HIV-2 gp120 during the course of this PhD. To improve this analysis of where the antibodies bind in relation to the shape of the molecule, it would be useful to allow complex formation to take place and then purify the complexes using a size exclusion column prior to their examination by electron microscopy. This protocol would remove all the unbound material and give a clearer picture to analyse. Following this, Cryoelectronmicroscopy could be used to produce an averaged low resolution structure of the HIV-2 gp120 either in isolation or in complex with an antibody (Benjamin et al., 2004; Briggs et al., 2006).

Although, through the work carried out in this thesis we were able to determine that the purified HIV-2 rgp120 bound CD4 by ELISA, more defined information was difficult to obtain using Surface Plasmon Reasonance techniques. Further work could investigate measuring HIV-2 binding to CD4 using FACS analysis. However, this may be hindered by the lack of a well defined antibody recognising the CD4-binding site of HIV-2 Env.

It would be beneficial to obtain better functional information on the HIV-2 gp120 constructs. This would be assisted by the establishment of cell lines producing gp120s (based on HIV-2_{ROD} or patient-derived) with stronger binding affinities for CD4 and the development of antibodies that recognise CD4-induced epitopes (as for MAb 17b in HIV-1 (Rizzuto and Sodroski, 2000; Rizzuto et al., 1998; Thali et al., 1993)) that could be utilised as co-receptor mimics (Wu et al., 1996). Such mimics may aid co-crystallisation and structure determination as for HIV-1 gp120 (Huang et al., 2005; Kwong et al., 2000a; Kwong et al., 1998). Further, with such mimics circular dichroism and fluorescence spectroscopy could be used to investigate the conformational changes induced in HIV-2 gp120 on CD4 and co-receptor mimic binding. BIAcore Surface Plasmon Resonance and Isothermal titration calorimetry could be used to look at the binding energetics of these reactions. However, these experiments can only be carried out if greater amounts of protein are available (both in terms of quantities of protein and number of different constructs available).

4.7 Concluding Remarks

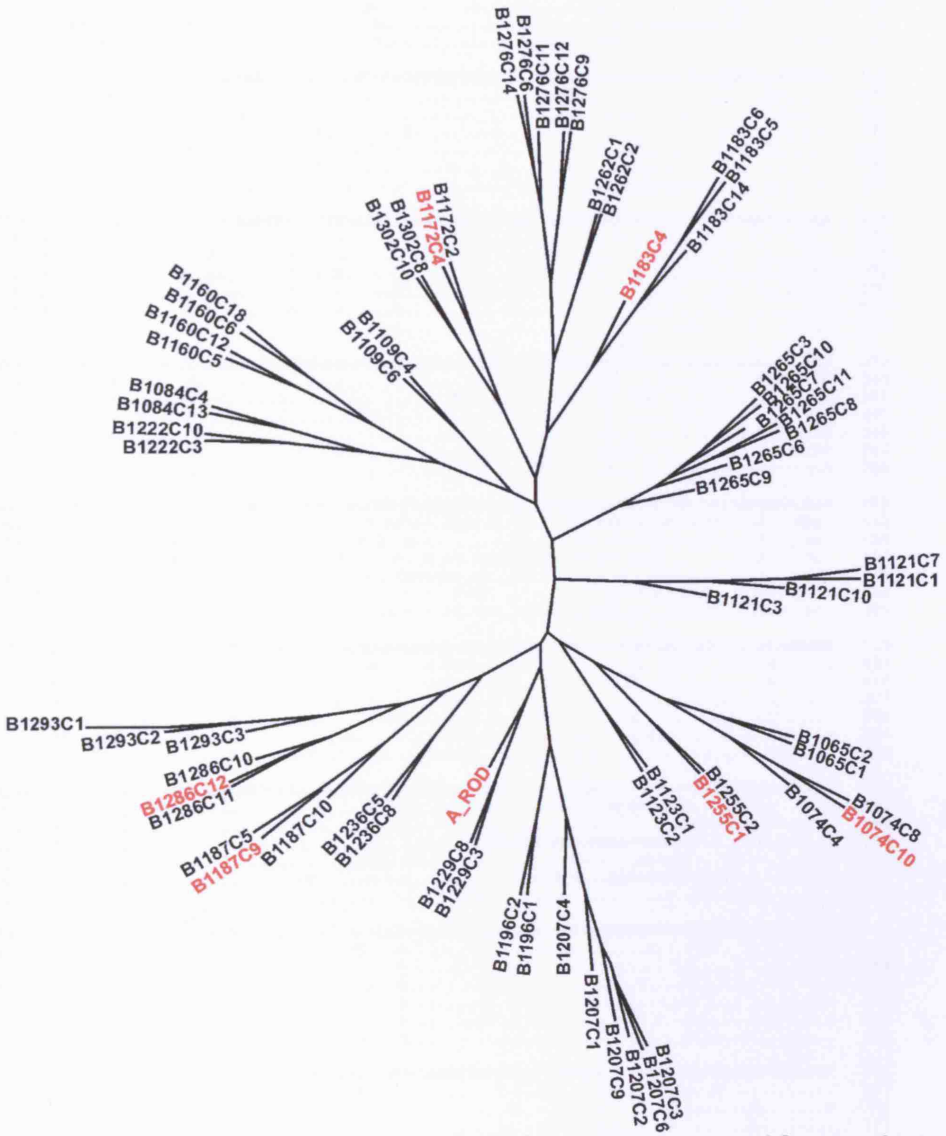
During the course of this PhD study four of the five original objectives were met. Firstly, a range of HIV-2 gp120 encoding constructs have been generated and shown to be expression competent. For HIV-2_{ROD} – based constructs a number of constitutively expressing CHO K1 cell lines have been established. Secondly, a purification procedure for one of the gp120 products has been developed. Thirdly, the purified product has been shown to be folded correctly, functional, and of a purity and quatity suitable for crystallisation trials. Finally, Although, crystallisation trials were unsuccessful, secondary tertiary structure analysis of the HIV-2 envelope glycoprotein by other techniques produced useful information.

Functional studies conducted on the gp120, related to the binding of CD4 and conformation specific antibodies suggest that the protein is correctly folded. Circular dichroism and fluorescence spectroscopy have recorded secondary structure information that is compatible with that reported for HIV-1 and HIV-2 glycoproteins (Chen et al., 2000; Hansen et al., 1996; Huang et al., 2005; Sourial et al., 2005). The oligomeric nature and stability of this protein has been probed by native PAGE-western blotting, dynamic light scattering, analytical ultracentrifugation and electron microscopy of isolated gp120 and gp120-complexed with monoclonal antibody. All these techniques indicated the existence of stable trimers of gp120 with a molecular weight in the range 290-350KDa depending on the method of analysis. These properties suggest that the characterised HIV-2_{ROD} gp120 would be a good immunogen for generation of antibodies for use in research and for consideration as part of a vaccine regimen, in addition to its crystallisation/structure determination trials.

Chapter 5

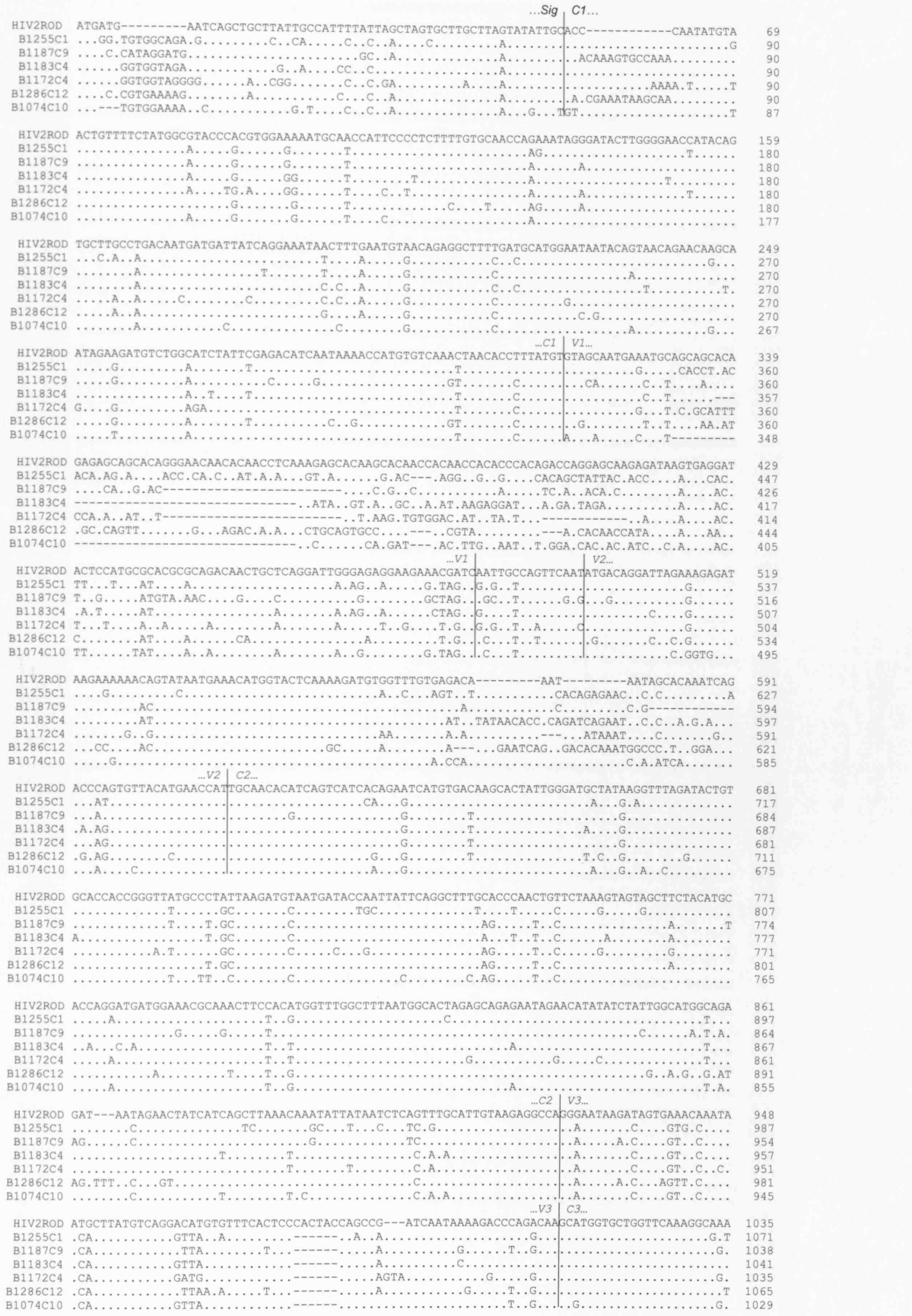
Appendices

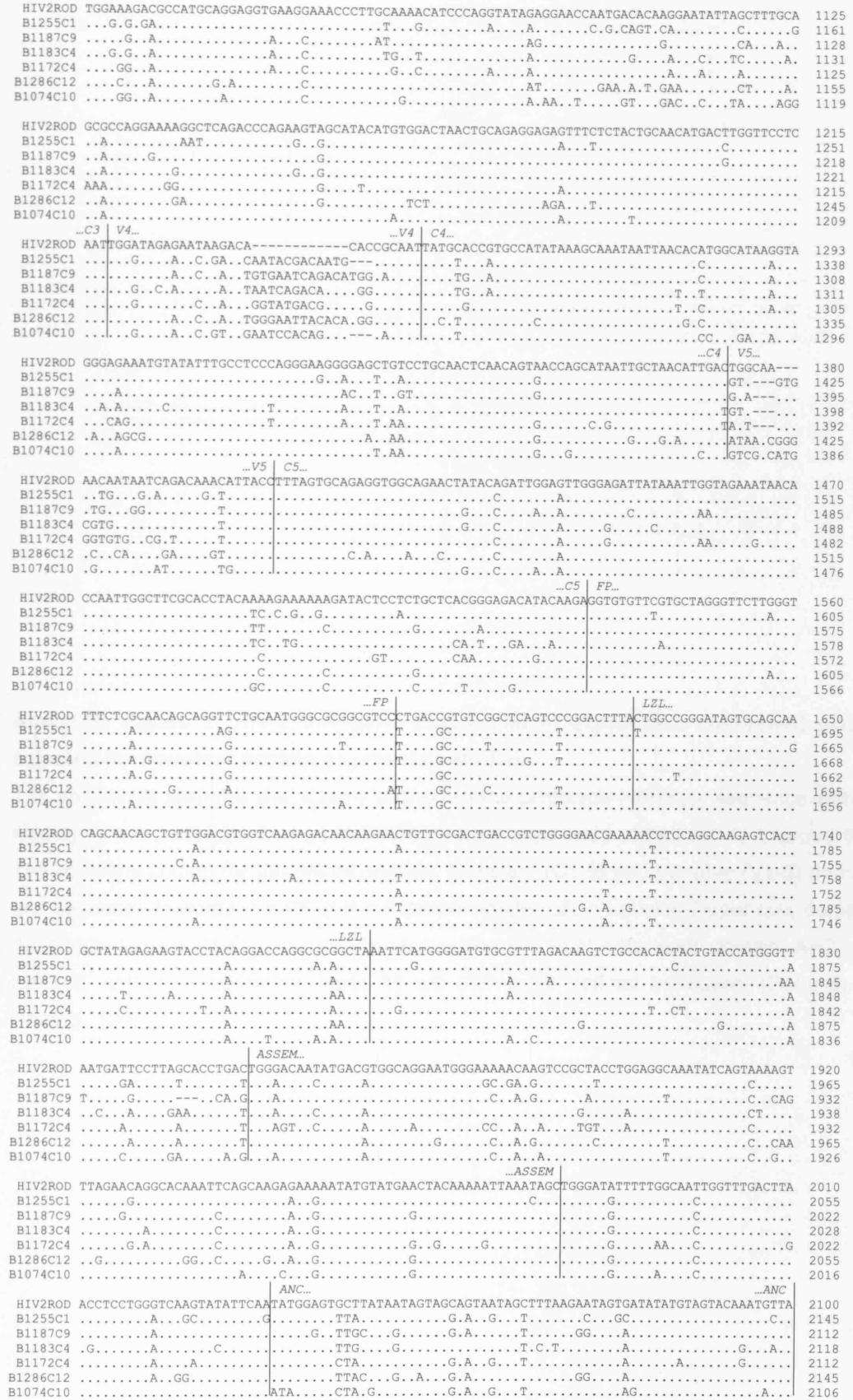
5 Appendices

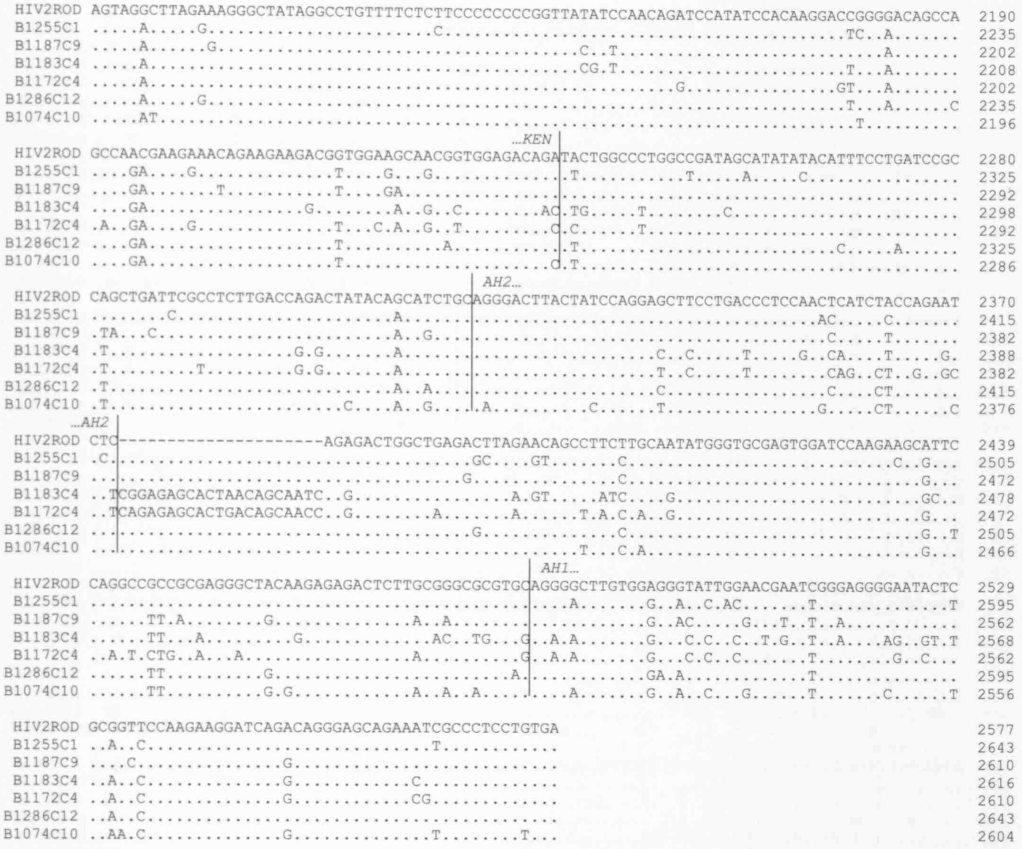


Appendix Figure 1: Phylogenetic analysis of HIV-2 *env*-genes rescued from patients enrolled in the Caio cohort

Analyses were performed using the neighbor-joining method (Pearson, Robins, and Zhang, 1999) contained within the Genetic Data Environment (GDE) suite of programmes (Smith et al., 1994). Initial analyses involving an extended dataset obtained from the HIV database (http:www.hiv.lanl.gov) demonstrated all Caio cohort-derived sequences to be of HIV-2A group (results not shown). The tree shown contains the prototype HIV-2_{ROD} and 66 Caio cohort-derived sequences and is the consensus of 100 trees generated. All sequences from a particular patient sample group together indicating that there was no cross-contamination during the DNA extraction, PCR and cloning processes. The positions of patient clones and HIV-2_{ROD}, used in the protein expression studies conducted in this report, are indicated in red.

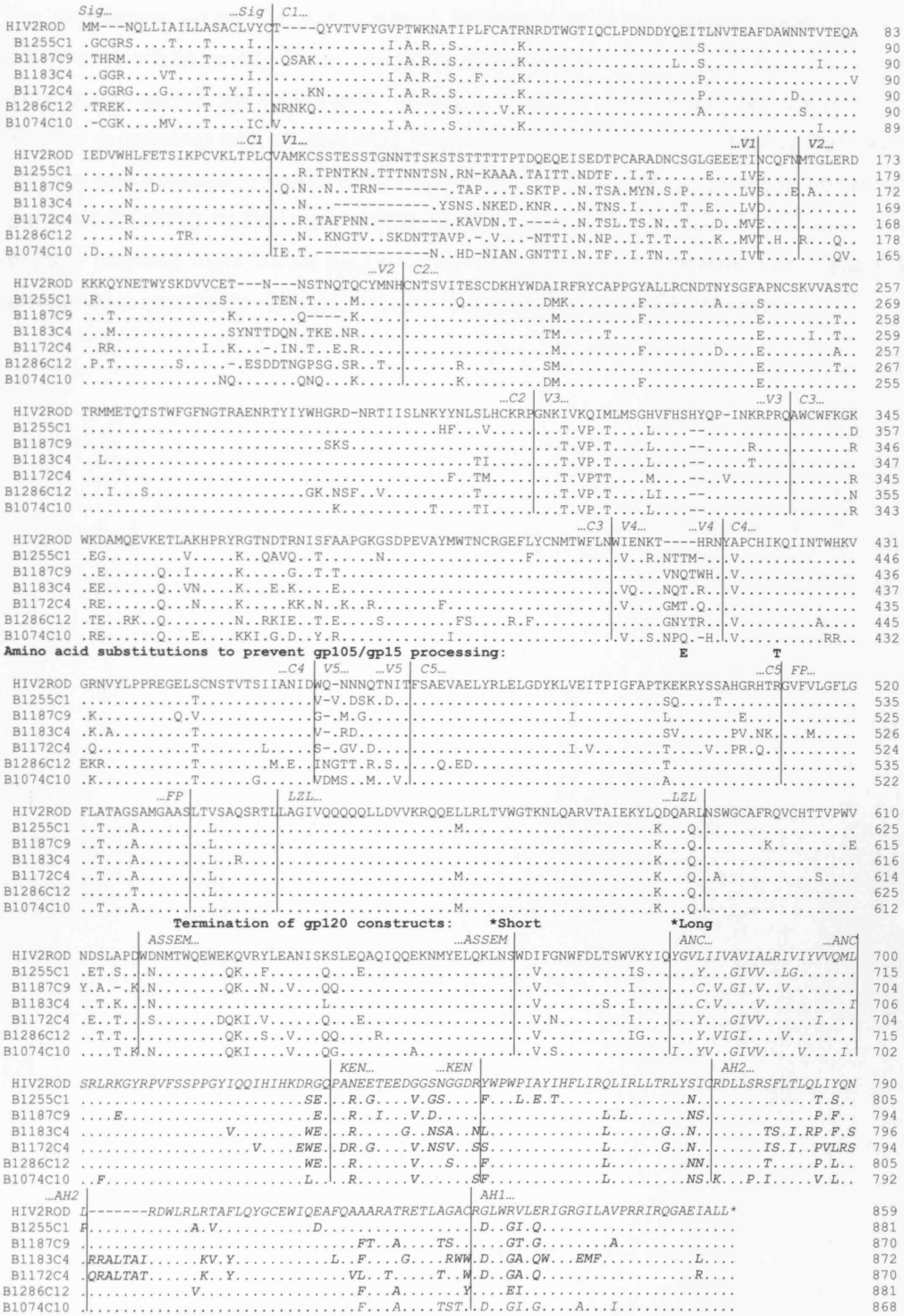

...Sig
C1...
...C1
V1...
...V1
V2...
...V2
C2...
...C2
V3...
...V3
C3...


HIV2ROD
B1255C1
B1187C9
B1183C4
B1172C4
B1286C12
B1074C10
...C3 | V4...
...V4 | C4...
...C4 | V5...
...V5 | C5...
...C5 | FP...
...FP
LZL...
...LZL
ASSEM...
...ASSEM
ANC...
...ANC
KEN...



Appendix Figure 2: HIV-2 *env*-gene sequences of expression competent pQ7-*env* clones selected for transient protein expression studies

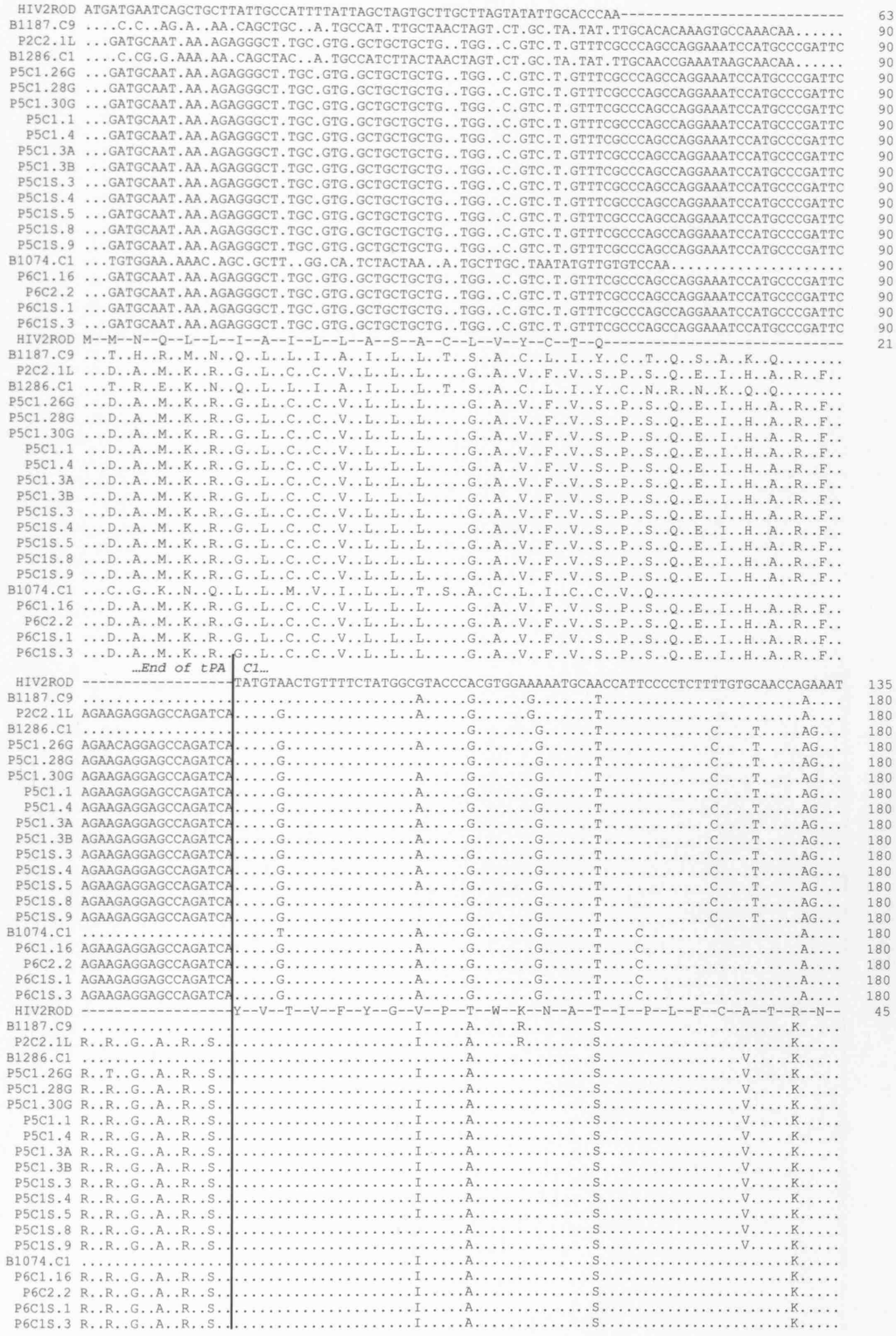
The six patient samples are shown aligned to the prototype HIV-2_{ROD} sequence with (.) indicating identity to the prototype and (-) indicating where gaps were introduced to improve the alignment. The positions coding for the signal peptide, Constant (C1-5) and Variable (V1-5) domains in gp105 and sequence features in gp36 (FP = Fusion Peptide, LZL = Leucine-Zipper-Like domain, ASSEM = Assembly domain, ANC = membrane anchor domain, KEN = Kennedy domain, AH2 = Amphipathic Helix 2, AH1 = Amphipathic Helix 1) are shown as defined previously (Douglas, Munro, and Daniels, 1997).

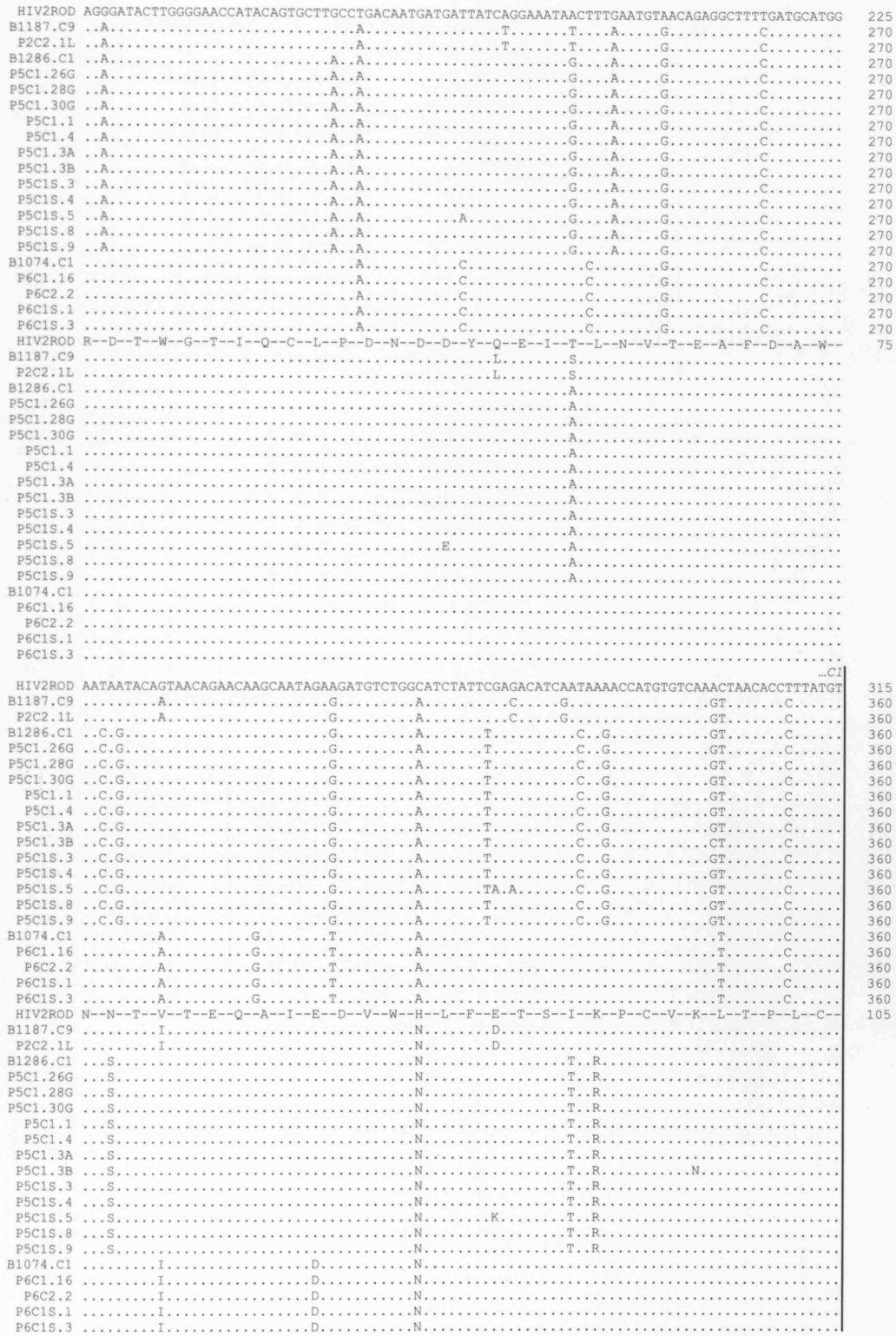


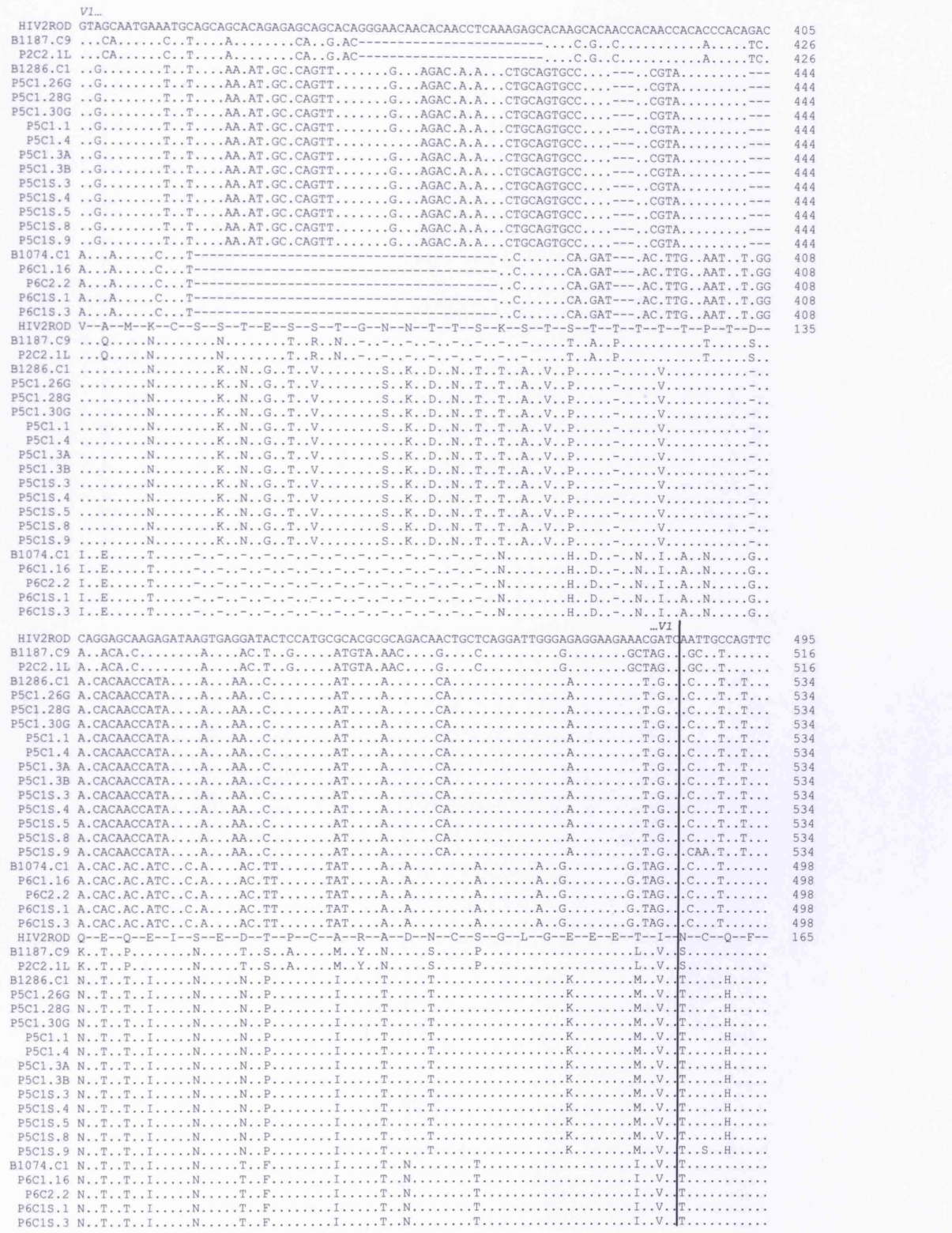
Appendix Figure 3: HIV-2 glycoprotein sequences of expression competent pQ7-*env* clones selected for transient protein expression studies

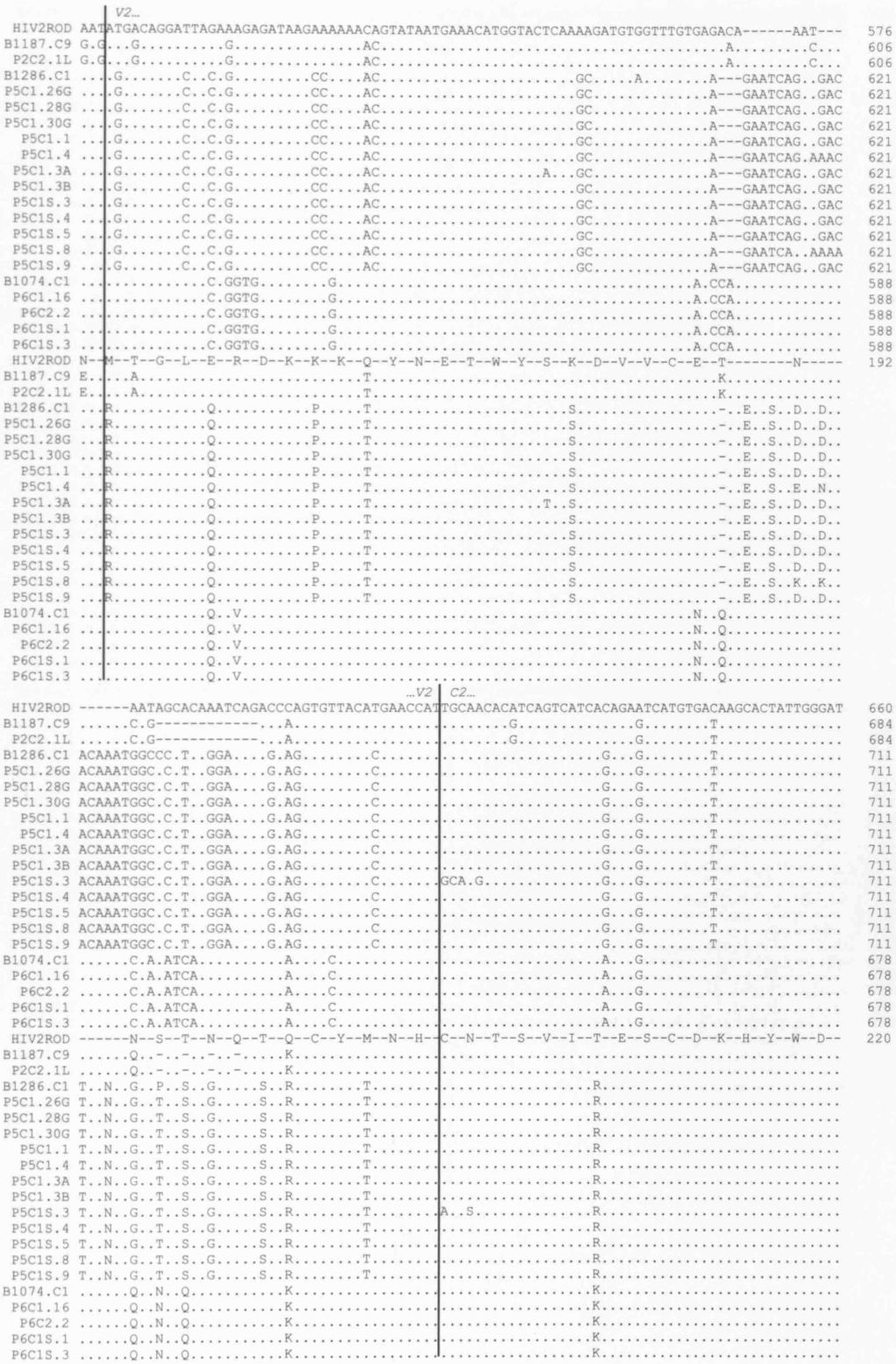
The six patient samples are shown aligned to the prototype HIV-2_{ROD} sequence with (.) indicating identity to the prototype and (-) indicating where gaps were introduced to improve the alignment. This figure is annotated as for Appendix Figure 2. In producing *env*-gene constructs for constitutive expression the coding sequences corresponding to the signal peptide and all gp36 sequences from

the membrane anchor to AH1 (shown in italics) were removed to produce the "long" (***Long**) constructs, subsequently "short" (***Short**) constructs truncated by 21 amino acids were generated. To produce gp105/gp15 cleavage defective mutants, two amino acid substitutions were inserted into the processing site as indicated (shown in bold). Sequence encoding the Fibritin trimerisation domain was inserted at positions corresponding to the C-termini of the gp120 constructs using the primers H2140FIB (L form) and NEWFIB (S form) in the PCR reactions (Table 2.1). A new signal peptide (derived from tissue plasminogen activator) and a hexa-His tag at the C-terminus of the glycoprotein constructs were introduced from the pEE14tPA2DCD4 vector.


…End of tPA
C1…




V1...
HIV2ROD GTAGCAATGAAATGCAGCAGCACAGAGAGCAGCACAGGGAACAACACAACCTCAAAGAGCACAAGCACAACCACAACCACACCCACAGAC 405
B1187.C9 ...CA.....C..T....A.....CA..G.AC---------- 426
P2C2.1L ...CA.....C..T....A.....CA..G.AC---------- 426
B1286.C1 ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1.26G ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1.28G ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1.30G ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1.1 ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1.4 ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1.3A ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1.3B ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1S.3 ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1S.4 ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1S.5 ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1S.8 ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
P5C1S.9 ..G.....T..T....AA.AT.GC.CAGTT.....G...AGAC.A.A...CTGCAGTGCC....---..CGTA.....--- 444
B1074.C1 A...A.....C...T----------..C.....CA.GAT---.AC.TTG..AAT..T.GG 408
P6C1.16 A...A.....C...T----------..C.....CA.GAT---.AC.TTG..AAT..T.GG 408
P6C2.2 A...A.....C...T----------..C.....CA.GAT---.AC.TTG..AAT..T.GG 408
P6C1S.1 A...A.....C...T----------..C.....CA.GAT---.AC.TTG..AAT..T.GG 408
P6C1S.3 A...A.....C...T----------..C.....CA.GAT---.AC.TTG..AAT..T.GG 408
HIV2ROD V--A--M--K--C--S--S--T--E--S--S--T--G--N--N--T--T--S--K--S--T--S--T--T--T--T--P--T--D-- 135
B1187.C9 ...Q.....N.....N.....T..R..N..-..-..-..-..-..-..-..-.....T..A..P.....T.....S..
P2C2.1L ...Q.....N.....N.....T..R..N..-..-..-..-..-..-..-..-.....T..A..P.....T.....S..
B1286.C1 .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1.26G .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1.28G .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1.30G .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1.1 .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1.4 .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1.3A .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1.3B .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1S.3 .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1S.4 .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1S.5 .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1S.8 .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
P5C1S.9 .....N.....K..N..G..T..V.....S..K..D..N..T..T..A..V..P.....-.....V.....-..
B1074.C1 I..E.....T.....-..-..-..-..-..-..-..-..-..-..-..-..-..-..N.....H..D..-..N..I..A..N.....G..
P6C1.16 I..E.....T.....-..-..-..-..-..-..-..-..-..-..-..-..-..-..N.....H..D..-..N..I..A..N.....G..
P6C2.2 I..E.....T.....-..-..-..-..-..-..-..-..-..-..-..-..-..-..N.....H..D..-..N..I..A..N.....G..
P6C1S.1 I..E.....T.....-..-..-..-..-..-..-..-..-..-..-..-..-..-..N.....H..D..-..N..I..A..N.....G..
P6C1S.3 I..E.....T.....-..-..-..-..-..-..-..-..-..-..-..-..-..-..N.....H..D..-..N..I..A..N.....G..
...V1
HIV2ROD CAGGAGCAAGAGATAAGTGAGGATACTCCATGCGCACGCGCAGACAACTGCTCAGGATTGGGAGAGGAAGAAACGATCAATTGCCAGTTC 495
B1187.C9 A..ACA.C.....A....AC.T..G.....ATGTA.AAC....G....C.....G.....GCTAG..GC..T..... 516
P2C2.1L A..ACA.C.....A....AC.T..G.....ATGTA.AAC....G....C.....G.....GCTAG..GC..T..... 516
B1286.C1 A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1.26G A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1.28G A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1.30G A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1.1 A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1.4 A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1.3A A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1.3B A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1S.3 A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1S.4 A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1S.5 A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1S.8 A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..C...T..T... 534
P5C1S.9 A.CACAACCATA....A...AA..C.....AT....A.....CA.....A.....T.G..CAA.T..T... 534
B1074.C1 A.CAC.AC.ATC..C.A....AC.TT.....TAT....A..A.....A.....A..G.....G.TAG..C...T..... 498
P6C1.16 A.CAC.AC.ATC..C.A....AC.TT.....TAT....A..A.....A.....A..G.....G.TAG..C...T..... 498
P6C2.2 A.CAC.AC.ATC..C.A....AC.TT.....TAT....A..A.....A.....A..G.....G.TAG..C...T..... 498
P6C1S.1 A.CAC.AC.ATC..C.A....AC.TT.....TAT....A..A.....A.....A..G.....G.TAG..C...T..... 498
P6C1S.3 A.CAC.AC.ATC..C.A....AC.TT.....TAT....A..A.....A.....A..G.....G.TAG..C...T..... 498
HIV2ROD Q--E--Q--E--I--S--E--D--T--P--C--A--R--A--D--N--C--S--G--L--G--E--E--E--T--I--N--C--Q--F-- 165
B1187.C9 K..T..P.....N.....T..S..A.....M..Y..N.....S.....P.....L..V..S.....
P2C2.1L K..T..P.....N.....T..S..A.....M..Y..N.....S.....P.....L..V..S.....
B1286.C1 N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1.26G N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1.28G N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1.30G N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1.1 N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1.4 N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1.3A N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1.3B N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1S.3 N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1S.4 N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1S.5 N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1S.8 N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T.....H.....
P5C1S.9 N..T..T..I.....N.....N..P.....I.....T.....T.....K.....M..V..T..S..H.....
B1074.C1 N..T..T..I.....N.....T..F.....I.....T..N.....T.....I..V..T.....
P6C1.16 N..T..T..I.....N.....T..F.....I.....T..N.....T.....I..V..T.....
P6C2.2 N..T..T..I.....N.....T..F.....I.....T..N.....T.....I..V..T.....
P6C1S.1 N..T..T..I.....N.....T..F.....I.....T..N.....T.....I..V..T.....
P6C1S.3 N..T..T..I.....N.....T..F.....I.....T..N.....T.....I..V..T.....


V2…
…V2
C2…

```
 HIV2ROD GCTATAAGGTTTAGATACTGTGCACCACCGGGTTATGCCCTATTAAGATGTAATGATACCAATTATTCAGGCTTTGCACCCAACTGTTCT  750
B1187.C9 .....G.............................T....T.GC.......C.........................AG.....T..C...  774
 P2C2.1L .....G.............................T....T.GC.......C.........................AG.....T..C...  774
B1286.C1 T.C..G........G.....................T.GC.....................................AG.....T..C...  801
P5C1.26G T.C..G........G.....................T.GC.....................................AG.....T..C...  801
P5C1.28G T.C..G........G.....................T.GC.....................................AG.....T..C...  801
P5C1.30G T.C..G........G.....................T.GC.....................................AG.....T..C...  801
  P5C1.1 T.C..G........G.....................T.GC.....................................AG.....T..C...  801
  P5C1.4 T.C..G........G.....................T.GC.....................................AG.....T..C...  801
 P5C1.3A T.C..G........G.....................T.GC.....................................AG.....T..C...  801
 P5C1.3B T.C..G........G.....................T.GC.....................................AG.....T..C...  801
 P5C1S.3 T.C..G........G.....................T.GC.....................................AG.....T..C...  801
 P5C1S.4 T.C..G........G.....................T.GC.....................................AG.....T..C...  801
 P5C1S.5 T.C..G........G.....................T.GC.....................................AG.....T..C...  801
 P5C1S.8 T.C..G........G.....................T.GC.....................................AG.....T..C...  801
 P5C1S.9 T.C..G........G.....................T.GC.....................................AG.....T..C...  801
B1074.C1 .A...G..A..C.......................T...TT..C.......C.............C........C.AG.....T..C...  768
 P6C1.16 .A...G..A..C.......................T...TT..C.......C.............C........C.AG.....T..C...  768
  P6C2.2 .A...G..A..C.......................T...TT..C.......C.............C........C.AG.....T..C...  768
 P6C1S.1 .A...G..A..C.......................T...TT..C.......C.............C........C.AG.....T..C...  768
 P6C1S.3 .A...G..A..C.......................T...TT..C.......C.............C........C.AG.....T..C...  768
 HIV2ROD A--I--R--F--R--Y--C--A--P--P--G--Y--A--L--L--R--C--N--D--T--N--Y--S--G--F--A--P--N--C--S--  250
B1187.C9 ...M..............................F.....................................E.....................
 P2C2.1L ...M..............................F.....................................E.....................
B1286.C1 S..M.............................................................................E.....................
P5C1.26G S..M.............................................................................E.....................
P5C1.28G S..M.............................................................................E.....................
P5C1.30G S..M.............................................................................E.....................
  P5C1.1 S..M.............................................................................E.....................
  P5C1.4 S..M.............................................................................E.....................
 P5C1.3A S..M.............................................................................E.....................
 P5C1.3B S..M.............................................................................E.....................
 P5C1S.3 S..M.............................................................................E.....................
 P5C1S.4 S..M.............................................................................E.....................
 P5C1S.5 S..M.............................................................................E.....................
 P5C1S.8 S..M.............................................................................E.....................
 P5C1S.9 S..M.............................................................................E.....................
B1074.C1 D..M..............................F.....................................E.....................
 P6C1.16 D..M..............................F.....................................E.....................
  P6C2.2 D..M..............................F.....................................E.....................
 P6C1S.1 D..M..............................F.....................................E.....................
 P6C1S.3 D..M..............................F.....................................E.....................

 HIV2ROD AAAGTAGTAGCTTCTACATGCACCAGGATGATGGAAACGCAAACTTCCACATGGTTTGGCTTTAATGGCACTAGAGCAGAGAATAGAACA  840
B1187.C9 ............A.......T..............G.....G.....T.....................................  864
 P2C2.1L ............A.......T..............G.....G.....T.....................................  864
B1286.C1 ............A.....................A.........T....T..G.....................................  891
P5C1.26G ............A.....................A.........T....T..G.....................................  891
P5C1.28G ............A.....................A.........T....T..G.....................................  891
P5C1.30G ............A.....................A.........T....T..G.....................................  891
  P5C1.1 ............A.....................A.........T....T..G.....................................  891
  P5C1.4 ............A.....................A.........T....T..G.....................................  891
 P5C1.3A ............A.....................A.........T....T..G.....................................  891
 P5C1.3B ............A.....................A.........T....T..G.....................................  891
 P5C1S.3 ............A.....................A.........T....T..G.....................................  891
 P5C1S.4 ............A.....................A.........T....T..G.....................................  891
 P5C1S.5 ............A.....................A.........T....T..G.....................................  891
 P5C1S.8 ............A.....................A.........T....T..G.....................................  891
 P5C1S.9 ............A.....................A.........T....T..G.....................................  891
B1074.C1 ........................A.....................T..G.............................A.........  858
 P6C1.16 ........................A.....................T..G.............................A.........  858
  P6C2.2 ........................A.....................T..G.............................A.........  858
 P6C1S.1 ........................A.....................T..G.............................A.........  858
 P6C1S.3 ........................A.....................T..G.............................A.........  858
 HIV2ROD K--V--V--A--S--T--C--T--R--M--M--E--T--Q--T--S--T--W--F--G--F--N--G--T--R--A--E--N--R--T--  280
B1187.C9 ............T.....................................................................
 P2C2.1L ............T.....................................................................
B1286.C1 ............T.................I...........S.....................................................
P5C1.26G ............T.................I...........S.....................................................
P5C1.28G ............T.................I...........S.....................................................
P5C1.30G ............T.................I...........S.....................................................
  P5C1.1 ............T.................I...........S.....................................................
  P5C1.4 ............T.................I...........S.....................................................
 P5C1.3A ............T.................I...........S.....................................................
 P5C1.3B ............T.................I...........S.....................................................
 P5C1S.3 ............T.................I...........S.....................................................
 P5C1S.4 ............T.................I...........S.....................................................
 P5C1S.5 ............T.................I...........S.....................................................
 P5C1S.8 ............T.................I...........S.....................................................
 P5C1S.9 ............T.................I...........S.....................................................
B1074.C1 .....................................................................................
 P6C1.16 .....................................................................................
  P6C2.2 .....................................................................................
 P6C1S.1 .....................................................................................
 P6C1S.3 .....................................................................................
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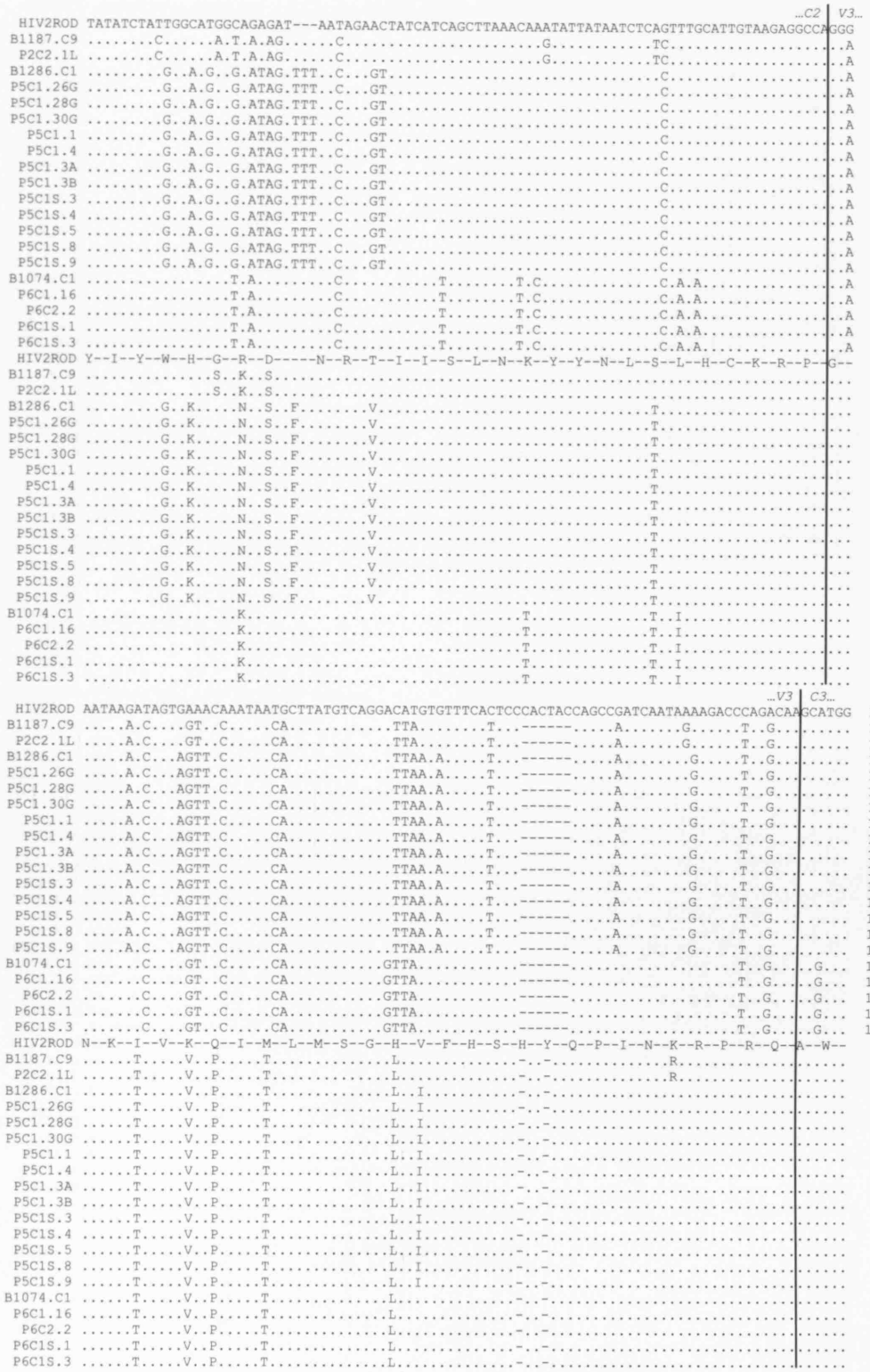
…C2 | V3…
HIV2ROD TATATCTATTGGCATGGCAGAGAT---AATAGAACTATCATCAGCTTAAACAAATATTATAATCTCAGTTTGCATTGTAAGAGGCCAGGG 927
B1187.C9 .....C.....A.T.A.AG.....C.....G.....TC.....A 954
P2C2.1L .....C.....A.T.A.AG.....C.....G.....TC.....A 954
B1286.C1 .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1.26G .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1.28G .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1.30G .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1.1 .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1.4 .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1.3A .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1.3B .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1S.3 .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1S.4 .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1S.5 .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1S.8 .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
P5C1S.9 .....G..A.G..G.ATAG.TTT..C...GT.....C.....A 981
B1074.C1 .....T.A.....C.....T.....T.C.....C.A.A.....A 948
P6C1.16 .....T.A.....C.....T.....T.C.....C.A.A.....A 948
P6C2.2 .....T.A.....C.....T.....T.C.....C.A.A.....A 948
P6C1S.1 .....T.A.....C.....T.....T.C.....C.A.A.....A 948
P6C1S.3 .....T.A.....C.....T.....T.C.....C.A.A.....A 948
HIV2ROD Y--I--Y--W--H--G--R--D-----N--R--T--I--I--S--L--N--K--Y--Y--N--L--S--L--H--C--K--R--P--G-- 309
B1187.C9 .....S..K..S..... ...
P2C2.1L .....S..K..S..... ...
B1286.C1 .....G..K.....N..S..F.....V.....T..... ...
P5C1.26G .....G..K.....N..S..F.....V.....T..... ...
P5C1.28G .....G..K.....N..S..F.....V.....T..... ...
P5C1.30G .....G..K.....N..S..F.....V.....T..... ...
P5C1.1 .....G..K.....N..S..F.....V.....T..... ...
P5C1.4 .....G..K.....N..S..F.....V.....T..... ...
P5C1.3A .....G..K.....N..S..F.....V.....T..... ...
P5C1.3B .....G..K.....N..S..F.....V.....T..... ...
P5C1S.3 .....G..K.....N..S..F.....V.....T..... ...
P5C1S.4 .....G..K.....N..S..F.....V.....T..... ...
P5C1S.5 .....G..K.....N..S..F.....V.....T..... ...
P5C1S.8 .....G..K.....N..S..F.....V.....T..... ...
P5C1S.9 .....G..K.....N..S..F.....V.....T..... ...
B1074.C1 .....K.....T.....T..I..... ...
P6C1.16 .....K.....T.....T..I..... ...
P6C2.2 .....K.....T.....T..I..... ...
P6C1S.1 .....K.....T.....T..I..... ...
P6C1S.3 .....K.....T.....T..I..... ...
…V3 | C3…
HIV2ROD AATAAGATAGTGAAACAAATAATGCTTATGTCAGGACATGTGTTTCACTCCCACTACCAGCCGATCAATAAAAGACCCAGACAAGCATGG 1017
B1187.C9 .....A.C....GT..C.....CA.....TTA.....T...------.....A.....G.....T..G..... 1038
P2C2.1L .....A.C....GT..C.....CA.....TTA.....T...------.....A.....G.....T..G..... 1038
B1286.C1 .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1.26G .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1.28G .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1.30G .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1.1 .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1.4 .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1.3A .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1.3B .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1S.3 .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1S.4 .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1S.5 .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1S.8 .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
P5C1S.9 .....A.C...AGTT.C.....CA.....TTAA.A.....T...------.....A.....G.....T..G..... 1065
B1074.C1 .....C....GT..C.....CA.....GTTA.....------.....T..G.....G... 1032
P6C1.16 .....C....GT..C.....CA.....GTTA.....------.....T..G.....G... 1032
P6C2.2 .....C....GT..C.....CA.....GTTA.....------.....T..G.....G... 1032
P6C1S.1 .....C....GT..C.....CA.....GTTA.....------.....T..G.....G... 1032
P6C1S.3 .....C....GT..C.....CA.....GTTA.....------.....T..G.....G... 1032
HIV2ROD N--K--I--V--K--Q--I--M--L--M--S--G--H--V--F--H--S--H--Y--Q--P--I--N--K--R--P--R--Q--A--W-- 339
B1187.C9 .....T.....V..P.....T.....L.....-..-.....R..... .....
P2C2.1L .....T.....V..P.....T.....L.....-..-.....R..... .....
B1286.C1 .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1.26G .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1.28G .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1.30G .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1.1 .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1.4 .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1.3A .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1.3B .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1S.3 .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1S.4 .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1S.5 .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1S.8 .....T.....V..P.....T.....L..I.....-..-..... .....
P5C1S.9 .....T.....V..P.....T.....L..I.....-..-..... .....
B1074.C1 .....T.....V..P.....T.....L.....-..-..... .....
P6C1.16 .....T.....V..P.....T.....L.....-..-..... .....
P6C2.2 .....T.....V..P.....T.....L.....-..-..... .....
P6C1S.1 .....T.....V..P.....T.....L.....-..-..... .....
P6C1S.3 .....T.....V..P.....T.....L.....-..-..... .....

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  HIV2ROD TGCTGGTTCAAAGGCAAATGGAAAGACGCCATGCAGGAGGTGAAGGAAACCCTTGCAAAACATCCCAGGTATAGAGGAACCAATGACACA 1107
B1187.C9 ................G......G..A..............A...C.........AT..................AG.............. 1128
 P2C2.1L ................G......G..A..............A...C.........AT..................AG.............. 1128
B1286.C1 ..................T....C...A.......G.A........C.............................AT.......GAA.A.T. 1155
P5C1.26G ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
P5C1.28G ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
P5C1.30G ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
  P5C1.1 ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
  P5C1.4 ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
 P5C1.3A ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
 P5C1.3B ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
 P5C1S.3 ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
 P5C1S.4 ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
 P5C1S.5 ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
 P5C1S.8 ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
 P5C1S.9 ..................T....C...A.......G..........C.............................AT.......GAA.A.T. 1155
B1074.C1 ................G.....GG..A........A.........C...........G.................A.AA..T.....GT... 1122
 P6C1.16 ................G.....GG..A........A.........C...........G.................A.AA..T.....GT... 1122
  P6C2.2 ................G.....GG..A........A.........C...........G.................A.AA..T.....GT... 1122
 P6C1S.1 ................G.....GG..A........A.........C...........G.................A.AA..T.....GT... 1122
 P6C1S.3 ................G.....GG..A........A.........C...........G.................A.AA..T.....GT... 1122
  HIV2ROD C--W--F--K--G--K--W--K--D--A--M--Q--E--V--K--E--T--L--A--K--H--P--R--Y--R--G--T--N--D--T--  369
B1187.C9 ..............R........E.....................Q........I.................K.....................
 P2C2.1L ..............R........E.....................Q........I.................K.....................
B1286.C1 ..............N.....T..E........R..K........Q.............................N........R..K..I..
P5C1.26G ..............N.....T..E........R...........Q.............................N........R..K..I..
P5C1.28G ..............N.....T..E........R...........Q.............................N........R..K..I..
P5C1.30G ..............N.....T..E........R...........Q.............................N........R..K..I..
  P5C1.1 ..............N.....T..E........R...........Q.............................N........R..K..I..
  P5C1.4 ..............N.....T..E........R...........Q.............................N........R..K..I..
 P5C1.3A ..............N.....T..E........R...........Q.............................N........R..K..I..
 P5C1.3B ..............N.....T..E........R...........Q.............................N........R..K..I..
 P5C1S.3 ..............N.....T..E........R...........Q.............................N........R..K..I..
 P5C1S.4 ..............N.....T..E........R...........Q.............................N........R..K..I..
 P5C1S.5 ..............N.....T..E........R...........Q.............................N........R..K..I..
 P5C1S.8 ..............N.....T..E........R...........Q.............................N........R..K..I..
 P5C1S.9 ..............N.....T..E........R...........Q.............................N........R..K..I..
B1074.C1 ..............R.....R..E.....................Q...........E.................K..K..I.....G.....
 P6C1.16 ..............R.....R..E.....................Q...........E.................K..K..I.....G.....
  P6C2.2 ..............R.....R..E.....................Q...........E.................K..K..I.....G.....
 P6C1S.1 ..............R.....R..E.....................Q...........E.................K..K..I.....G.....
 P6C1S.3 ..............R.....R..E.....................Q...........E.................K..K..I.....G.....

  HIV2ROD AGGAATATTAGCTTTGCAGCGCCAGGAAAAGGCTCAGACCCAGAAGTAGCATACATGTGGACTAACTGCAGAGGAGAGTTTCTCTACTGC 1197
B1187.C9 G.........CA...A....A.....G.....................G........................................ 1218
 P2C2.1L G.........CA...A....A.....G.....................G........................................ 1218
B1286.C1 GAA.......CT....A...A........GA.................G.........TCT.............AGA...T........ 1245
P5C1.26G GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
P5C1.28G GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
P5C1.30G GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
  P5C1.1 GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
  P5C1.4 GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
 P5C1.3A GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
 P5C1.3B GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
 P5C1S.3 GAA.......CT....A...A........GA.................G.........TCT.................A..CT........ 1245
 P5C1S.4 GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
 P5C1S.5 GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
 P5C1S.8 GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
 P5C1S.9 GAA.......CT....A...A........GA.................G.........TCT.................A...T........ 1245
B1074.C1 GAC..C...TA....AGG..A.....................................A.....................A........T... 1212
 P6C1.16 GAC..C...TA....AGG..A.....................................A.....................A........T... 1212
  P6C2.2 GAC..C...TA....AGG..A.....................................A.....................A........T... 1212
 P6C1S.1 GAC..C...TA....AGG..A.....................................A.....................A........T... 1212
 P6C1S.3 GAC..C...TA....AGG..A.....................................A.....................A........T... 1212
  HIV2ROD R--N--I--S--F--A--A--P--G--K--G--S--D--P--E--V--A--Y--M--W--T--N--C--R--G--E--F--L--Y--C--  399
B1187.C9 G.........T.....T.....................................................................
 P2C2.1L G.........T.....T.....................................................................
B1286.C1 E........T.....E..............S.........................F..S..............R.....F........
P5C1.26G E........T.....E..............S.........................F..S.....................F........
P5C1.28G E........T.....E..............S.........................F..S.....................F........
P5C1.30G E........T.....E..............S.........................F..S.....................F........
  P5C1.1 E........T.....E..............S.........................F..S.....................F........
  P5C1.4 E........T.....E..............S.........................F..S.....................F........
 P5C1.3A E........T.....E..............S.........................F..S.....................F........
 P5C1.3B E........T.....E..............S.........................F..S.....................F........
 P5C1S.3 E........T.....E..............S.........................F..S.....................F........
 P5C1S.4 E........T.....E..............S.........................F..S.....................F........
 P5C1S.5 E........T.....E..............S.........................F..S.....................F........
 P5C1S.8 E........T.....E..............S.........................F..S.....................F........
 P5C1S.9 E........T.....E..............S.........................F..S.....................F........
B1074.C1 D........Y.....R.....................................I.....................................
 P6C1.16 D........Y.....R.....................................I.....................................
  P6C2.2 D........Y.....R.....................................I.....................................
 P6C1S.1 D........Y.....R.....................................I.....................................
 P6C1S.3 D........Y.....R.....................................I.....................................
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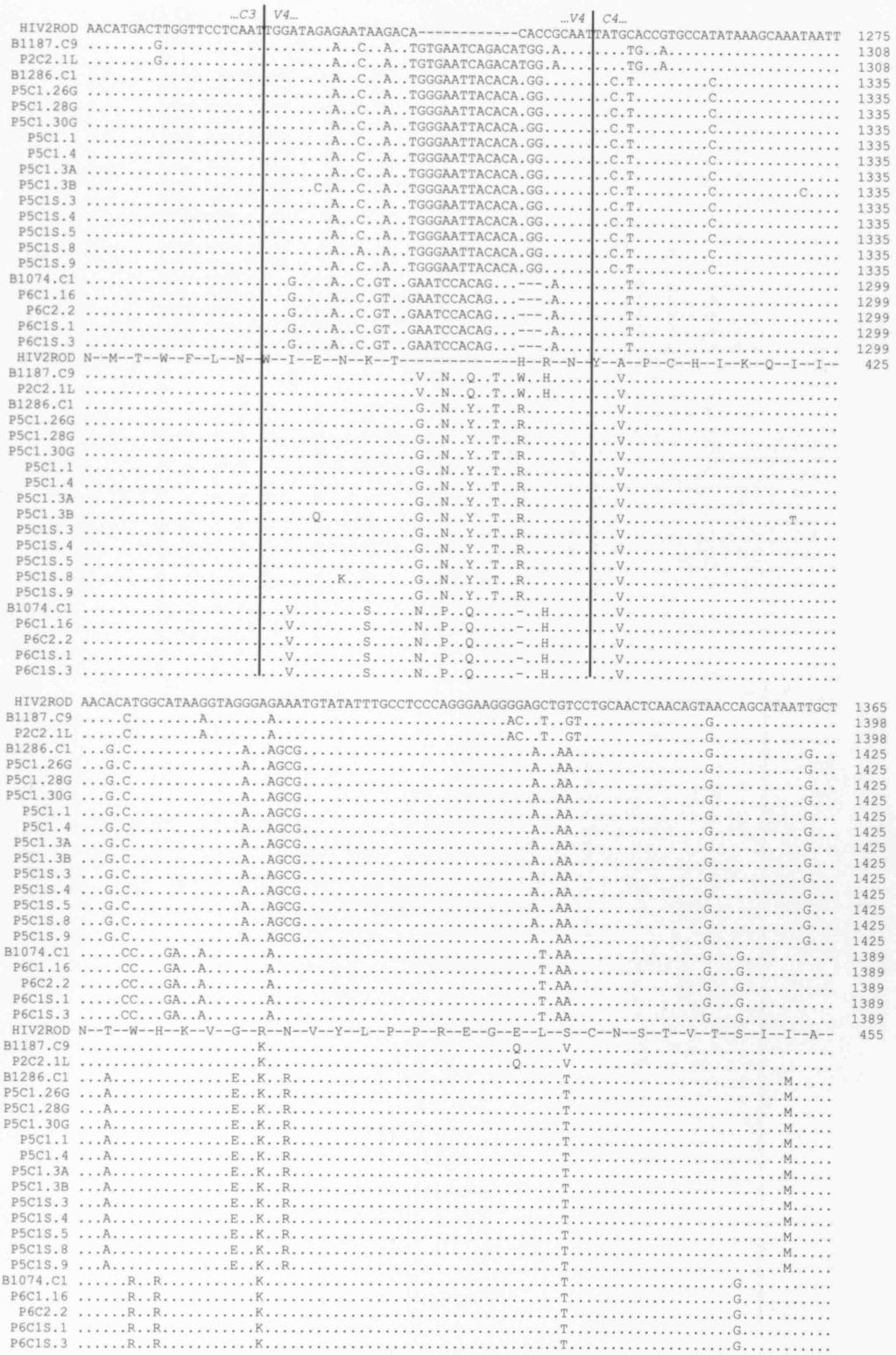
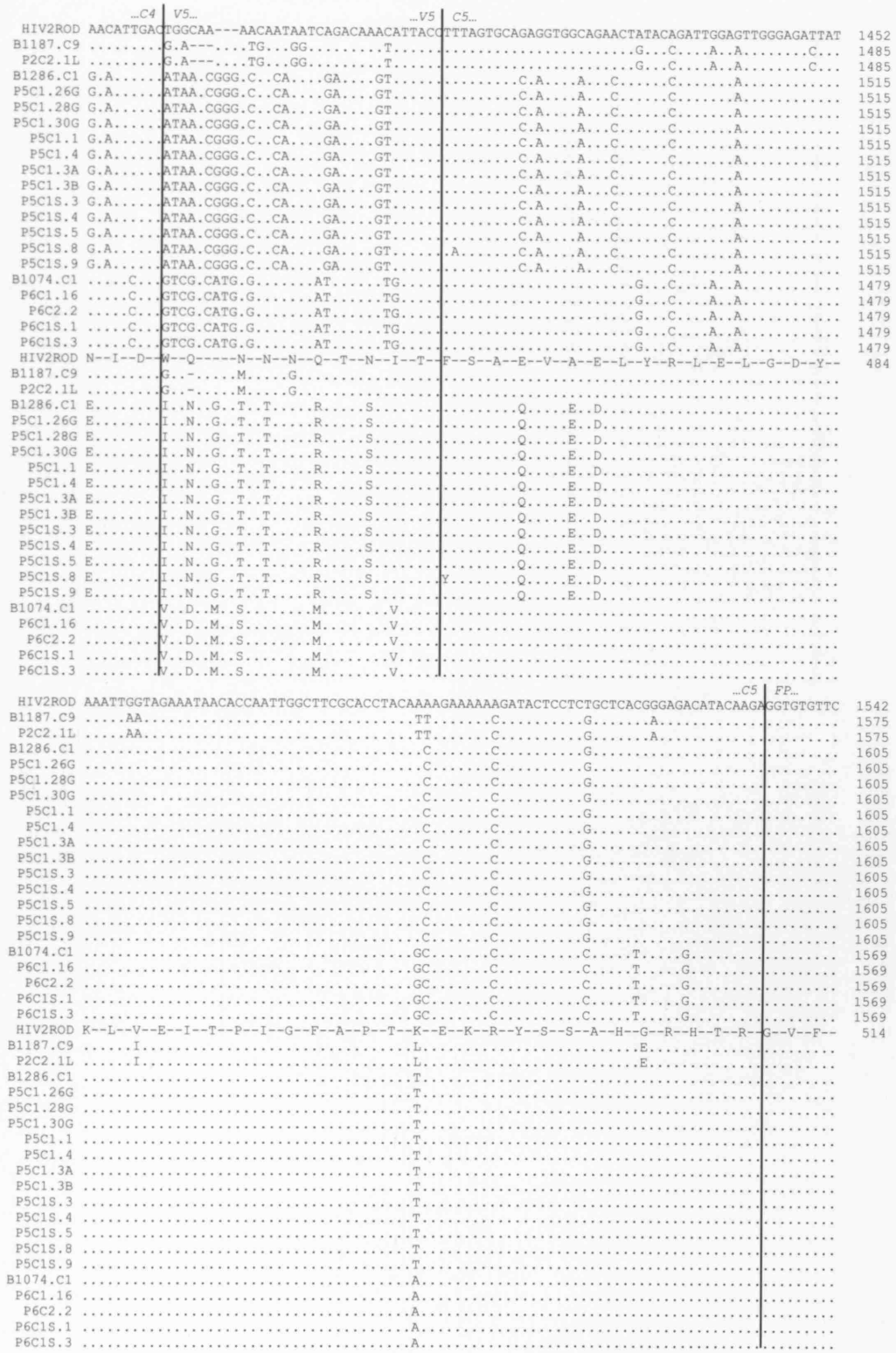
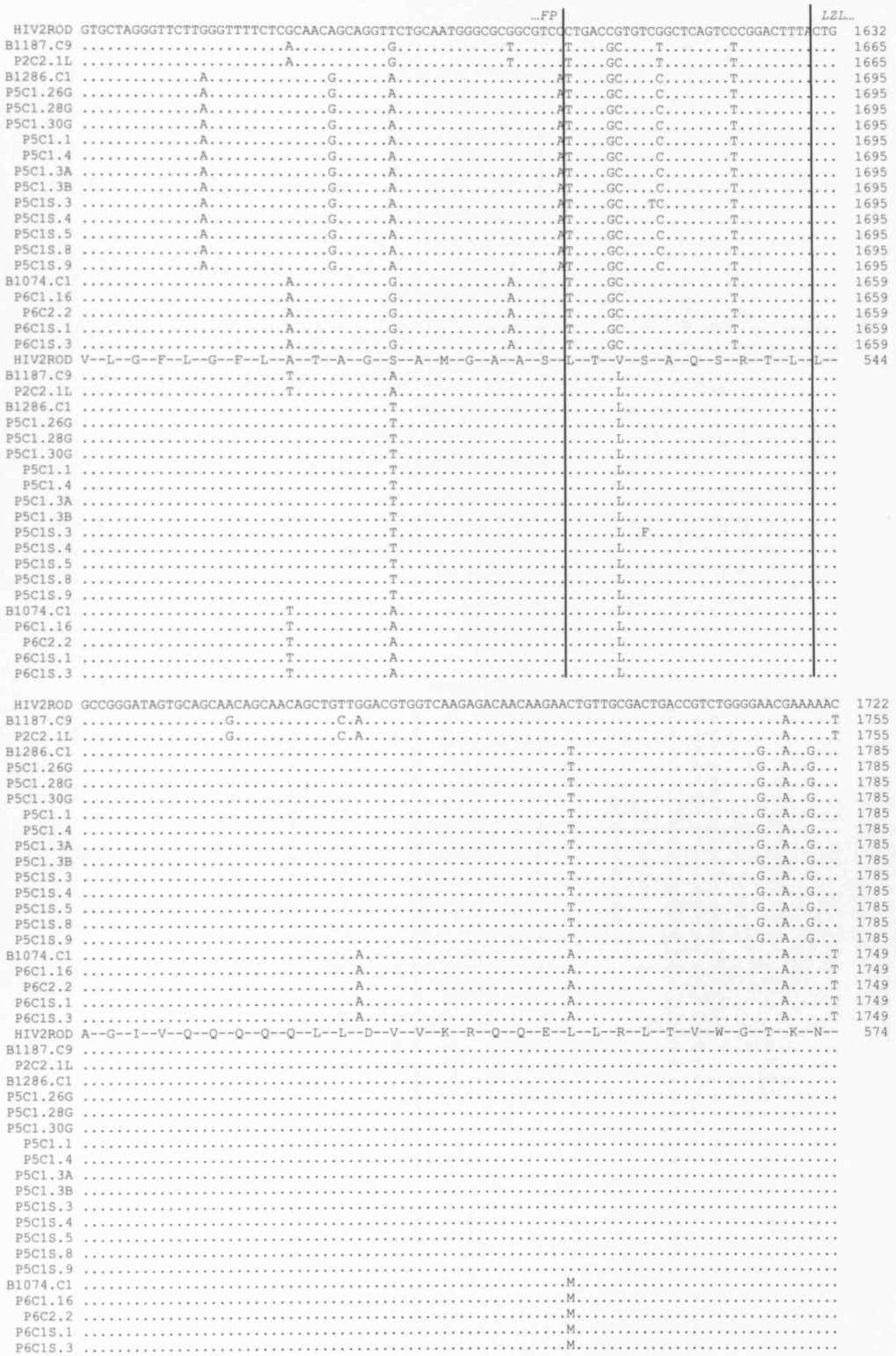
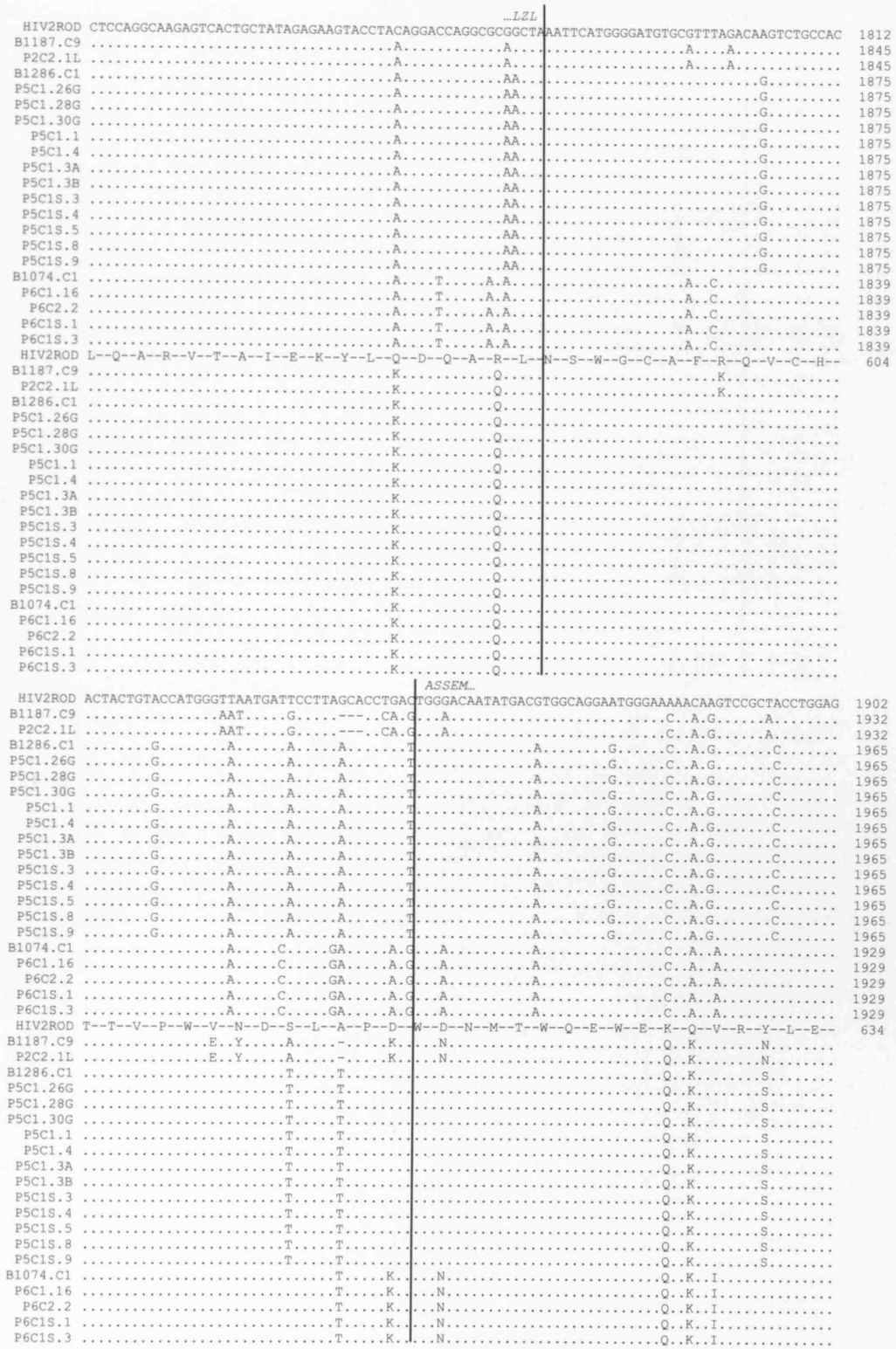
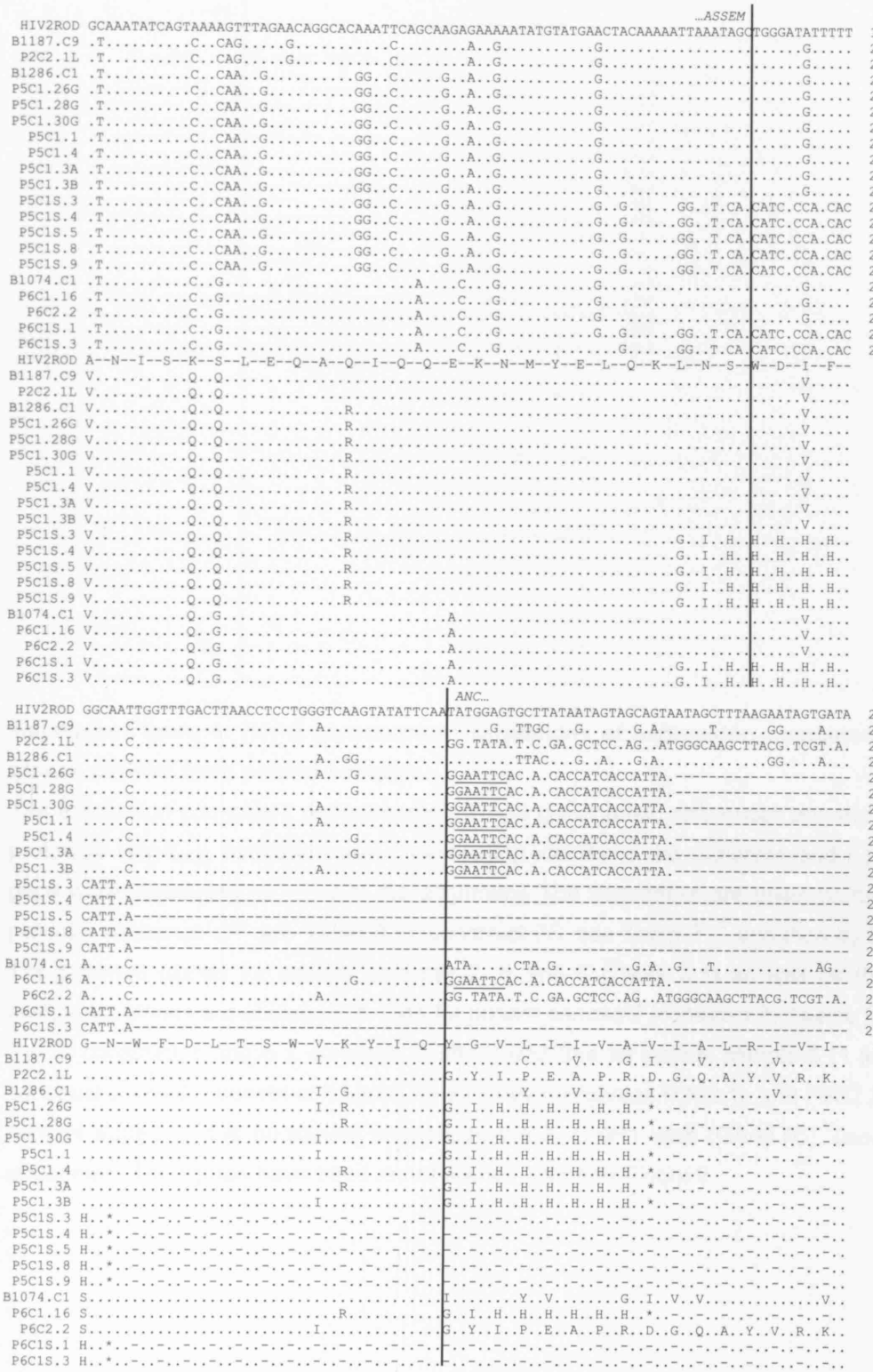
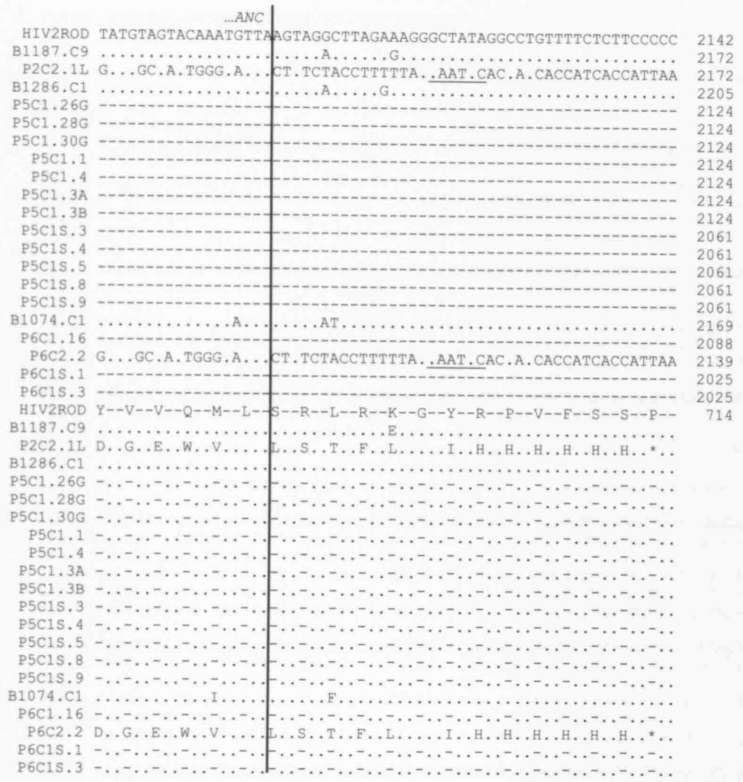

...C3
V4...
...V4
C4...
HIV2ROD AACATGACTTGGTTCCTCAATTGGATAGAGAATAAGACA----------CACCGCAATTATGCACCGTGCCATATAAAGCAAATAATT 1275
B1187.C9 ....G.....|.....A..C..A..TGTGAATCAGACATGG.A.....|....TG..A..... 1308
P2C2.1L ....G.....|.....A..C..A..TGTGAATCAGACATGG.A.....|....TG..A..... 1308
B1286.C1 .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1.26G .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1.28G .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1.30G .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1.1 .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1.4 .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1.3A .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1.3B .....|.....C.A..C..A..TGGGAATTACACA.GG.....|..C.T.....C.....C.... 1335
P5C1S.3 .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1S.4 .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1S.5 .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1S.8 .....|.....A..A..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
P5C1S.9 .....|.....A..C..A..TGGGAATTACACA.GG.....|..C.T.....C..... 1335
B1074.C1 .....|...G....A..C.GT..GAATCCACAG...---.A.....|....T..... 1299
P6C1.16 .....|...G....A..C.GT..GAATCCACAG...---.A.....|....T..... 1299
P6C2.2 .....|...G....A..C.GT..GAATCCACAG...---.A.....|....T..... 1299
P6C1S.1 .....|...G....A..C.GT..GAATCCACAG...---.A.....|....T..... 1299
P6C1S.3 .....|...G....A..C.GT..GAATCCACAG...---.A.....|....T..... 1299
HIV2ROD N--M--T--W--F--L--N--W--I--E--N--K--T----------H--R--N--Y--A--P--C--H--I--K--Q--I--I-- 425
B1187.C9 .....|.....V..N..Q..T..W..H.....|...V.....
P2C2.1L .....|.....V..N..Q..T..W..H.....|...V.....
B1286.C1 .....|.....G..N..Y..T..R.....|...V.....
P5C1.26G .....|.....G..N..Y..T..R.....|...V.....
P5C1.28G .....|.....G..N..Y..T..R.....|...V.....
P5C1.30G .....|.....G..N..Y..T..R.....|...V.....
P5C1.1 .....|.....G..N..Y..T..R.....|...V.....
P5C1.4 .....|.....G..N..Y..T..R.....|...V.....
P5C1.3A .....|.....G..N..Y..T..R.....|...V.....
P5C1.3B .....|.....Q.....G..N..Y..T..R.....|...V.....T.....
P5C1S.3 .....|.....G..N..Y..T..R.....|...V.....
P5C1S.4 .....|.....G..N..Y..T..R.....|...V.....
P5C1S.5 .....|.....G..N..Y..T..R.....|...V.....
P5C1S.8 .....|.....K.....G..N..Y..T..R.....|...V.....
P5C1S.9 .....|.....G..N..Y..T..R.....|...V.....
B1074.C1 .....|...V.....S.....N..P..Q.....-..H.....|...V.....
P6C1.16 .....|...V.....S.....N..P..Q.....-..H.....|...V.....
P6C2.2 .....|...V.....S.....N..P..Q.....-..H.....|...V.....
P6C1S.1 .....|...V.....S.....N..P..Q.....-..H.....|...V.....
P6C1S.3 .....|...V.....S.....N..P..Q.....-..H.....|...V.....
HIV2ROD AACACATGGCATAAGGTAGGGAGAAATGTATATTTGCCTCCCAGGGAAGGGGAGCTGTCCTGCAACTCAACAGTAACCAGCATAATTGCT 1365
B1187.C9 .....C.....A.....A.....AC..T..GT.....G..... 1398
P2C2.1L .....C.....A.....A.....AC..T..GT.....G..... 1398
B1286.C1 ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1.26G ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1.28G ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1.30G ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1.1 ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1.4 ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1.3A ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1.3B ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1S.3 ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1S.4 ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1S.5 ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1S.8 ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
P5C1S.9 ...G.C.....A..AGCG.....A..AA.....G.....G... 1425
B1074.C1 .....CC...GA..A.....A.....T.AA.....G...G..... 1389
P6C1.16 .....CC...GA..A.....A.....T.AA.....G...G..... 1389
P6C2.2 .....CC...GA..A.....A.....T.AA.....G...G..... 1389
P6C1S.1 .....CC...GA..A.....A.....T.AA.....G...G..... 1389
P6C1S.3 .....CC...GA..A.....A.....T.AA.....G...G..... 1389
HIV2ROD N--T--W--H--K--V--G--R--N--V--Y--L--P--P--R--E--G--E--L--S--C--N--S--T--V--T--S--I--I--A-- 455
B1187.C9 .....K.....Q.....V.....
P2C2.1L .....K.....Q.....V.....
B1286.C1 ...A.....E..K..R.....T.....M.....
P5C1.26G ...A.....E..K..R.....T.....M.....
P5C1.28G ...A.....E..K..R.....T.....M.....
P5C1.30G ...A.....E..K..R.....T.....M.....
P5C1.1 ...A.....E..K..R.....T.....M.....
P5C1.4 ...A.....E..K..R.....T.....M.....
P5C1.3A ...A.....E..K..R.....T.....M.....
P5C1.3B ...A.....E..K..R.....T.....M.....
P5C1S.3 ...A.....E..K..R.....T.....M.....
P5C1S.4 ...A.....E..K..R.....T.....M.....
P5C1S.5 ...A.....E..K..R.....T.....M.....
P5C1S.8 ...A.....E..K..R.....T.....M.....
P5C1S.9 ...A.....E..K..R.....T.....M.....
B1074.C1 .....R..R.....K.....T.....G.....
P6C1.16 .....R..R.....K.....T.....G.....
P6C2.2 .....R..R.....K.....T.....G.....
P6C1S.1 .....R..R.....K.....T.....G.....
P6C1S.3 .....R..R.....K.....T.....G.....

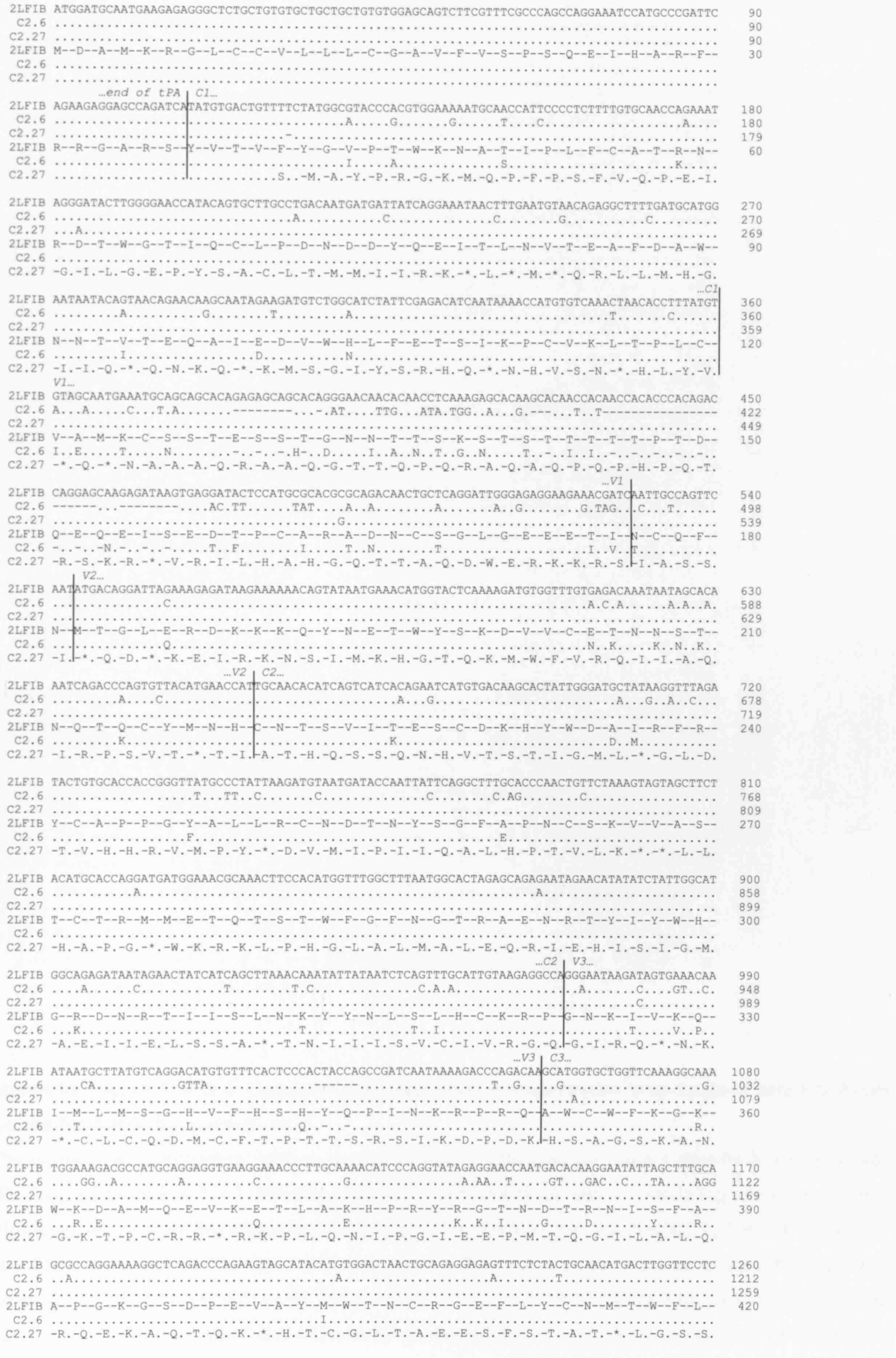

...C4
V5...
...V5
C5...
HIV2ROD AACATTGACTGGCAA---AACAATAATCAGACAAACATTACCTTTAGTGCAGAGGTGGCAGAACTATACAGATTGGAGTTGGGAGATTAT 1452
B1187.C9 .....G.A---....TG...GG.....T.....|.....G...C....A..A.....C... 1485
P2C2.1L .....G.A---....TG...GG.....T.....|.....G...C....A..A.....C... 1485
B1286.C1 G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1.26G G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1.28G G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1.30G G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1.1 G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1.4 G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1.3A G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1.3B G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1S.3 G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1S.4 G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1S.5 G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
P5C1S.8 G.A.....ATAA.CGGG.C..CA....GA....GT.....|A.....C.A....A...C.....C.....A..... 1515
P5C1S.9 G.A.....ATAA.CGGG.C..CA....GA....GT.....|.....C.A....A...C.....C.....A..... 1515
B1074.C1 .....C...GTCG.CATG.G.....AT.....TG.....|.....G...C....A..A..... 1479
P6C1.16 .....C...GTCG.CATG.G.....AT.....TG.....|.....G...C....A..A..... 1479
P6C2.2 .....C...GTCG.CATG.G.....AT.....TG.....|.....G...C....A..A..... 1479
P6C1S.1 .....C...GTCG.CATG.G.....AT.....TG.....|.....G...C....A..A..... 1479
P6C1S.3 .....C...GTCG.CATG.G.....AT.....TG.....|.....G...C....A..A..... 1479
HIV2ROD N--I--D--W--Q-----N--N--N--Q--T--N--I--T--F--S--A--E--V--A--E--L--Y--R--L--E--L--G--D--Y-- 484
B1187.C9 .....G..-.....M.....G.....|.....
P2C2.1L .....G..-.....M.....G.....|.....
B1286.C1 E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1.26G E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1.28G E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1.30G E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1.1 E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1.4 E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1.3A E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1.3B E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1S.3 E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1S.4 E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1S.5 E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
P5C1S.8 E.....I..N..G..T..T.....R.....S.....|Y.....Q.....E..D.....
P5C1S.9 E.....I..N..G..T..T.....R.....S.....|.....Q.....E..D.....
B1074.C1 .....V..D..M..S.....M.....V.....|.....
P6C1.16 .....V..D..M..S.....M.....V.....|.....
P6C2.2 .....V..D..M..S.....M.....V.....|.....
P6C1S.1 .....V..D..M..S.....M.....V.....|.....
P6C1S.3 .....V..D..M..S.....M.....V.....|.....
...C5
FP...
HIV2ROD AAATTGGTAGAAATAACACCAATTGGCTTCGCACCTACAAAAGAAAAAAGATACTCCTCTGCTCACGGGAGACATACAAGAGGTGTGTTC 1542
B1187.C9 .....AA.....TT.....C.....G.....A.....|..... 1575
P2C2.1L .....AA.....TT.....C.....G.....A.....|..... 1575
B1286.C1 .....C.....C.....G.....|..... 1605
P5C1.26G .....C.....C.....G.....|..... 1605
P5C1.28G .....C.....C.....G.....|..... 1605
P5C1.30G .....C.....C.....G.....|..... 1605
P5C1.1 .....C.....C.....G.....|..... 1605
P5C1.4 .....C.....C.....G.....|..... 1605
P5C1.3A .....C.....C.....G.....|..... 1605
P5C1.3B .....C.....C.....G.....|..... 1605
P5C1S.3 .....C.....C.....G.....|..... 1605
P5C1S.4 .....C.....C.....G.....|..... 1605
P5C1S.5 .....C.....C.....G.....|..... 1605
P5C1S.8 .....C.....C.....G.....|..... 1605
P5C1S.9 .....C.....C.....G.....|..... 1605
B1074.C1 .....GC.....C.....C.....T.....G.....|..... 1569
P6C1.16 .....GC.....C.....C.....T.....G.....|..... 1569
P6C2.2 .....GC.....C.....C.....T.....G.....|..... 1569
P6C1S.1 .....GC.....C.....C.....T.....G.....|..... 1569
P6C1S.3 .....GC.....C.....C.....T.....G.....|..... 1569
HIV2ROD K--L--V--E--I--T--P--I--G--F--A--P--T--K--E--K--R--Y--S--S--A--H--G--R--H--T--R--G--V--F-- 514
B1187.C9 .....I.....L.....E.....|.....
P2C2.1L .....I.....L.....E.....|.....
B1286.C1 .....T.....|.....
P5C1.26G .....T.....|.....
P5C1.28G .....T.....|.....
P5C1.30G .....T.....|.....
P5C1.1 .....T.....|.....
P5C1.4 .....T.....|.....
P5C1.3A .....T.....|.....
P5C1.3B .....T.....|.....
P5C1S.3 .....T.....|.....
P5C1S.4 .....T.....|.....
P5C1S.5 .....T.....|.....
P5C1S.8 .....T.....|.....
P5C1S.9 .....T.....|.....
B1074.C1 .....A.....|.....
P6C1.16 .....A.....|.....
P6C2.2 .....A.....|.....
P6C1S.1 .....A.....|.....
P6C1S.3 .....A.....|.....

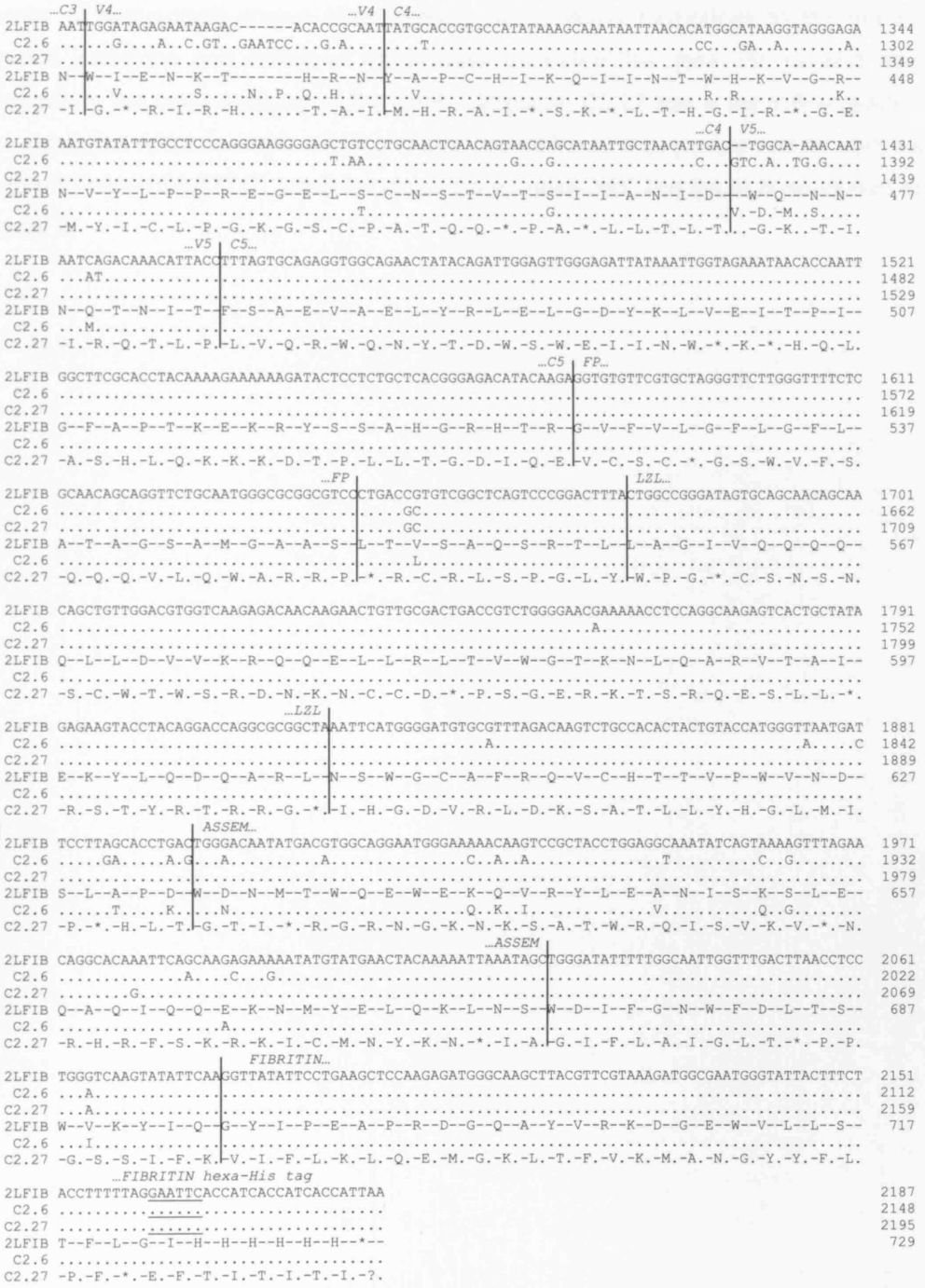

...FP
LZL...
HIV2ROD GTGCTAGGGTTCTTGGGTTTTCTCGCAACAGCAGGTTCTGCAATGGGCGCGGCGTCCCTGACCGTGTCGGCTCAGTCCCGGACTTTACTG 1632
B1187.C9 ....A.....G.....T.....T....GC....T.....T.....A... 1665
P2C2.1L ....A.....G.....T.....T....GC....T.....T.....A... 1665
B1286.C1 ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1.26G ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1.28G ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1.30G ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1.1 ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1.4 ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1.3A ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1.3B ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1S.3 ...A.....G.....A.....AT....GC...TC.....T.....A... 1695
P5C1S.4 ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1S.5 ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1S.8 ...A.....G.....A.....AT....GC....C.....T.....A... 1695
P5C1S.9 ...A.....G.....A.....AT....GC....C.....T.....A... 1695
B1074.C1 ....A.....G.....A.....T....GC.....T.....A... 1659
P6C1.16 ....A.....G.....A.....T....GC.....T.....A... 1659
P6C2.2 ....A.....G.....A.....T....GC.....T.....A... 1659
P6C1S.1 ....A.....G.....A.....T....GC.....T.....A... 1659
P6C1S.3 ....A.....G.....A.....T....GC.....T.....A... 1659
HIV2ROD V--L--G--F--L--G--F--L--A--T--A--G--S--A--M--G--A--A--S--L--T--V--S--A--Q--S--R--T--L--L-- 544
B1187.C9 ....T.....A.....L.....
P2C2.1L ....T.....A.....L.....
B1286.C1 ....T.....T.....L.....
P5C1.26G ....T.....T.....L.....
P5C1.28G ....T.....T.....L.....
P5C1.30G ....T.....T.....L.....
P5C1.1 ....T.....T.....L.....
P5C1.4 ....T.....T.....L.....
P5C1.3A ....T.....T.....L.....
P5C1.3B ....T.....T.....L.....
P5C1S.3 ....T.....T.....L..F.....
P5C1S.4 ....T.....T.....L.....
P5C1S.5 ....T.....T.....L.....
P5C1S.8 ....T.....T.....L.....
P5C1S.9 ....T.....T.....L.....
B1074.C1 ....T.....A.....L.....
P6C1.16 ....T.....A.....L.....
P6C2.2 ....T.....A.....L.....
P6C1S.1 ....T.....A.....L.....
P6C1S.3 ....T.....A.....L.....
HIV2ROD GCCGGGATAGTGCAGCAACAGCAACAGCTGTTGGACGTGGTCAAGAGACAACAAGAACTGTTGCGACTGACCGTCTGGGGAACGAAAAAC 1722
B1187.C9 ....G.....C.A.....A.....T 1755
P2C2.1L ....G.....C.A.....A.....T 1755
B1286.C1 .....T.....G..A..G... 1785
P5C1.26G .....T.....G..A..G... 1785
P5C1.28G .....T.....G..A..G... 1785
P5C1.30G .....T.....G..A..G... 1785
P5C1.1 .....T.....G..A..G... 1785
P5C1.4 .....T.....G..A..G... 1785
P5C1.3A .....T.....G..A..G... 1785
P5C1.3B .....T.....G..A..G... 1785
P5C1S.3 .....T.....G..A..G... 1785
P5C1S.4 .....T.....G..A..G... 1785
P5C1S.5 .....T.....G..A..G... 1785
P5C1S.8 .....T.....G..A..G... 1785
P5C1S.9 .....T.....G..A..G... 1785
B1074.C1 ....A.....A.....A.....T 1749
P6C1.16 ....A.....A.....A.....T 1749
P6C2.2 ....A.....A.....A.....T 1749
P6C1S.1 ....A.....A.....A.....T 1749
P6C1S.3 ....A.....A.....A.....T 1749
HIV2ROD A--G--I--V--Q--Q--Q--Q--Q--L--L--D--V--V--K--R--Q--Q--E--L--L--R--L--T--V--W--G--T--K--N-- 574
B1187.C9 .....
P2C2.1L .....
B1286.C1 .....
P5C1.26G .....
P5C1.28G .....
P5C1.30G .....
P5C1.1 .....
P5C1.4 .....
P5C1.3A .....
P5C1.3B .....
P5C1S.3 .....
P5C1S.4 .....
P5C1S.5 .....
P5C1S.8 .....
P5C1S.9 .....
B1074.C1 .....M.....
P6C1.16 .....M.....
P6C2.2 .....M.....
P6C1S.1 .....M.....
P6C1S.3 .....M.....


...LZL
ASSEM...


...ASSEM
ANC...


...ANC
HIV2ROD TATGTAGTACAAATGTTAAGTAGGCTTAGAAAGGGCTATAGGCCTGTTTTCTCTTCCCCC 2142
B1187.C9 .....A.....G..... 2172
P2C2.1L G...GC.A.TGGG.A...CT.TCTACCTTTTTA..AAT.CAC.A.CACCATCACCATTAA 2172
B1286.C1 .....A.....G..... 2205
P5C1.26G ---------- 2124
P5C1.28G ---------- 2124
P5C1.30G ---------- 2124
P5C1.1 ---------- 2124
P5C1.4 ---------- 2124
P5C1.3A ---------- 2124
P5C1.3B ---------- 2124
P5C1S.3 ---------- 2061
P5C1S.4 ---------- 2061
P5C1S.5 ---------- 2061
P5C1S.8 ---------- 2061
P5C1S.9 ---------- 2061
B1074.C1 .....A.....AT..... 2169
P6C1.16 ---------- 2088
P6C2.2 G...GC.A.TGGG.A...CT.TCTACCTTTTTA..AAT.CAC.A.CACCATCACCATTAA 2139
P6C1S.1 ---------- 2025
P6C1S.3 ---------- 2025
HIV2ROD Y--V--V--Q--M--L--S--R--L--R--K--G--Y--R--P--V--F--S--S--P-- 714
B1187.C9 .....E.....
P2C2.1L D..G..E..W..V.....L..S..T..F..L.....I..H..H..H..H..H..H..*..
B1286.C1 .....
P5C1.26G -..-..-..-


2LFIB
C2.6
C2.27
…end of tPA
C1…
V1…
…C1
…V1
V2…
…V2
C2…
…C2
V3…
…V3
C3…

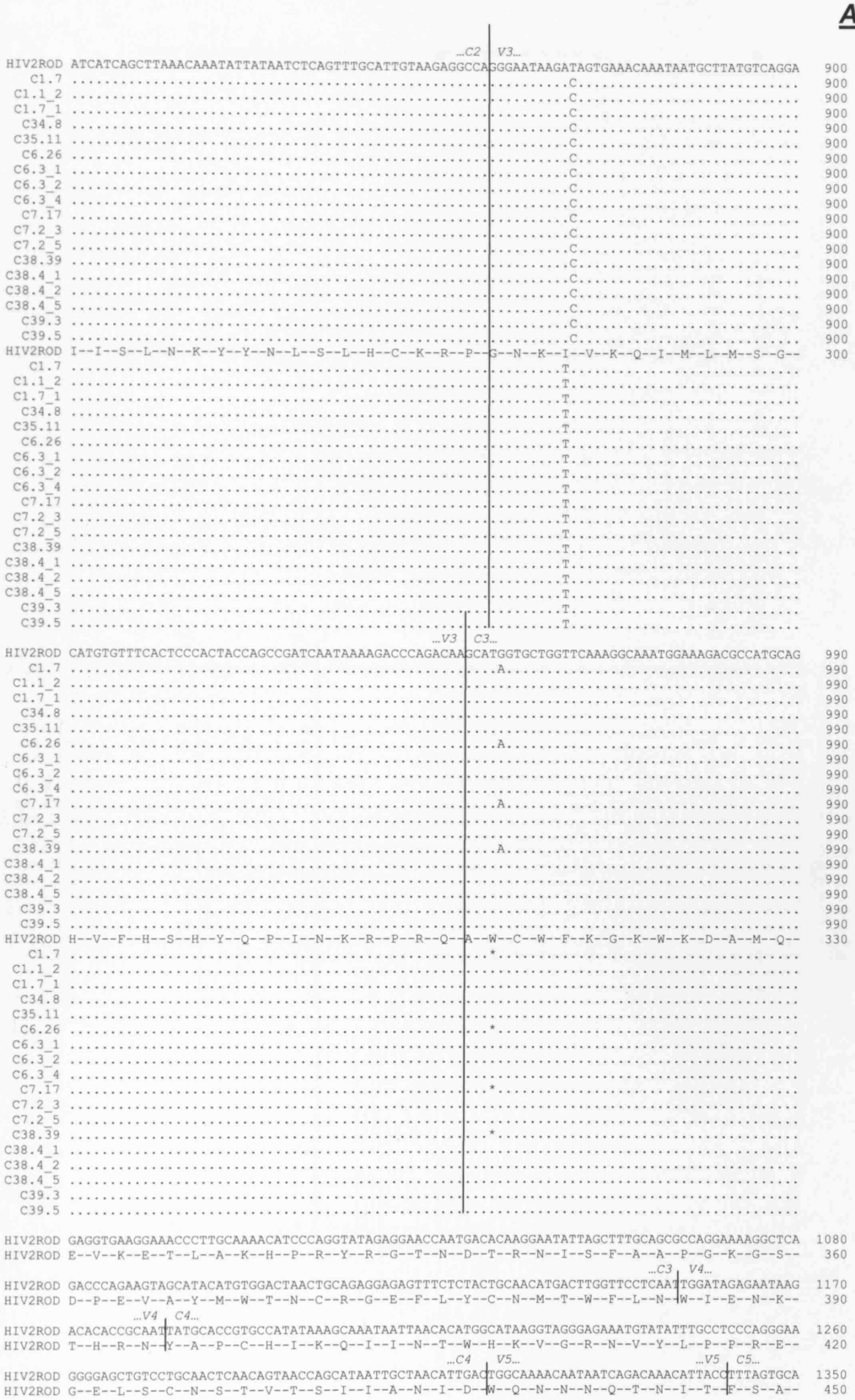


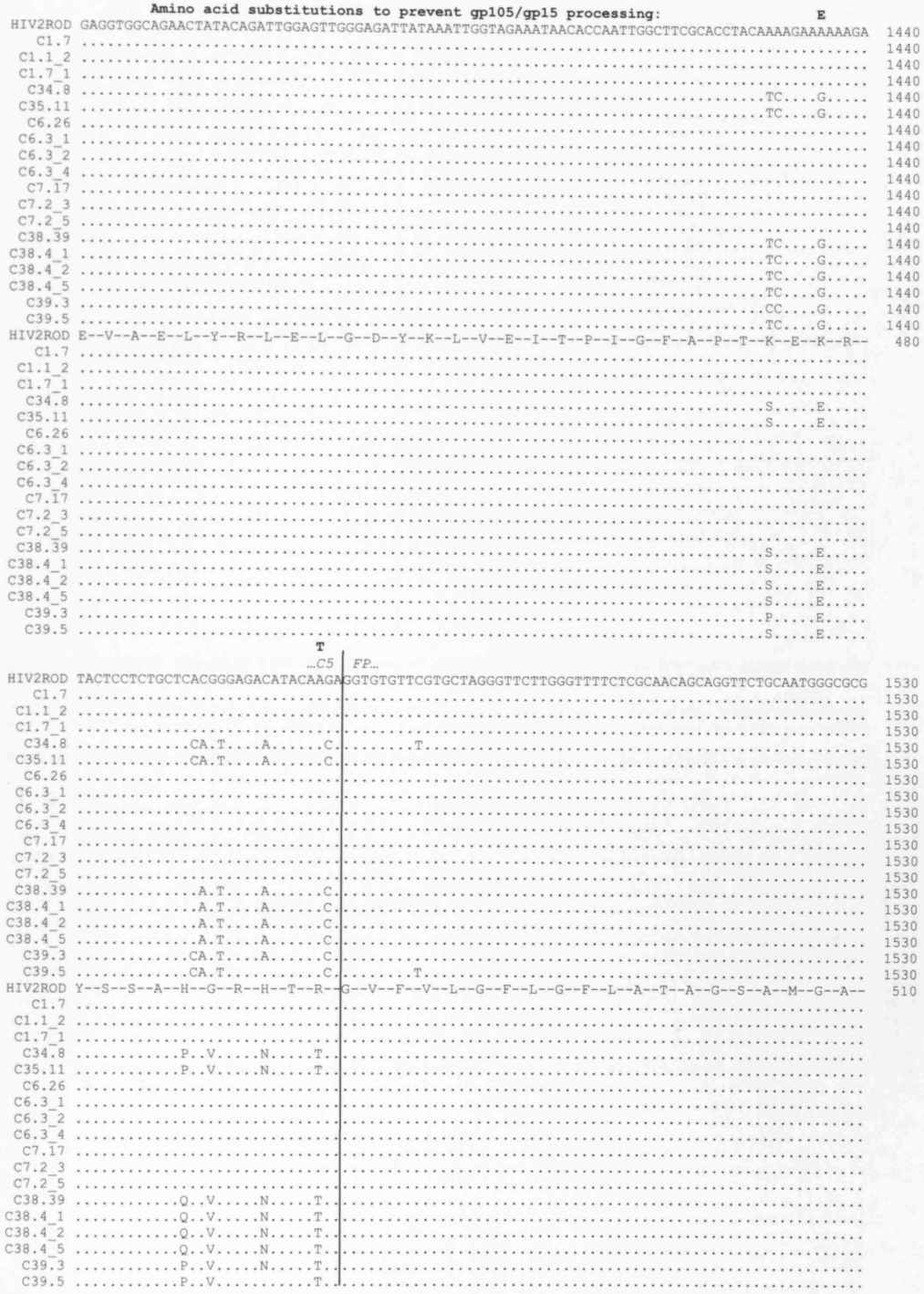
Appendix Figure 5: HIV-2 glycoprotein sequences of expression incompetent pEE14tPA-*env* clones from HIV-2_{ROD} construct 2

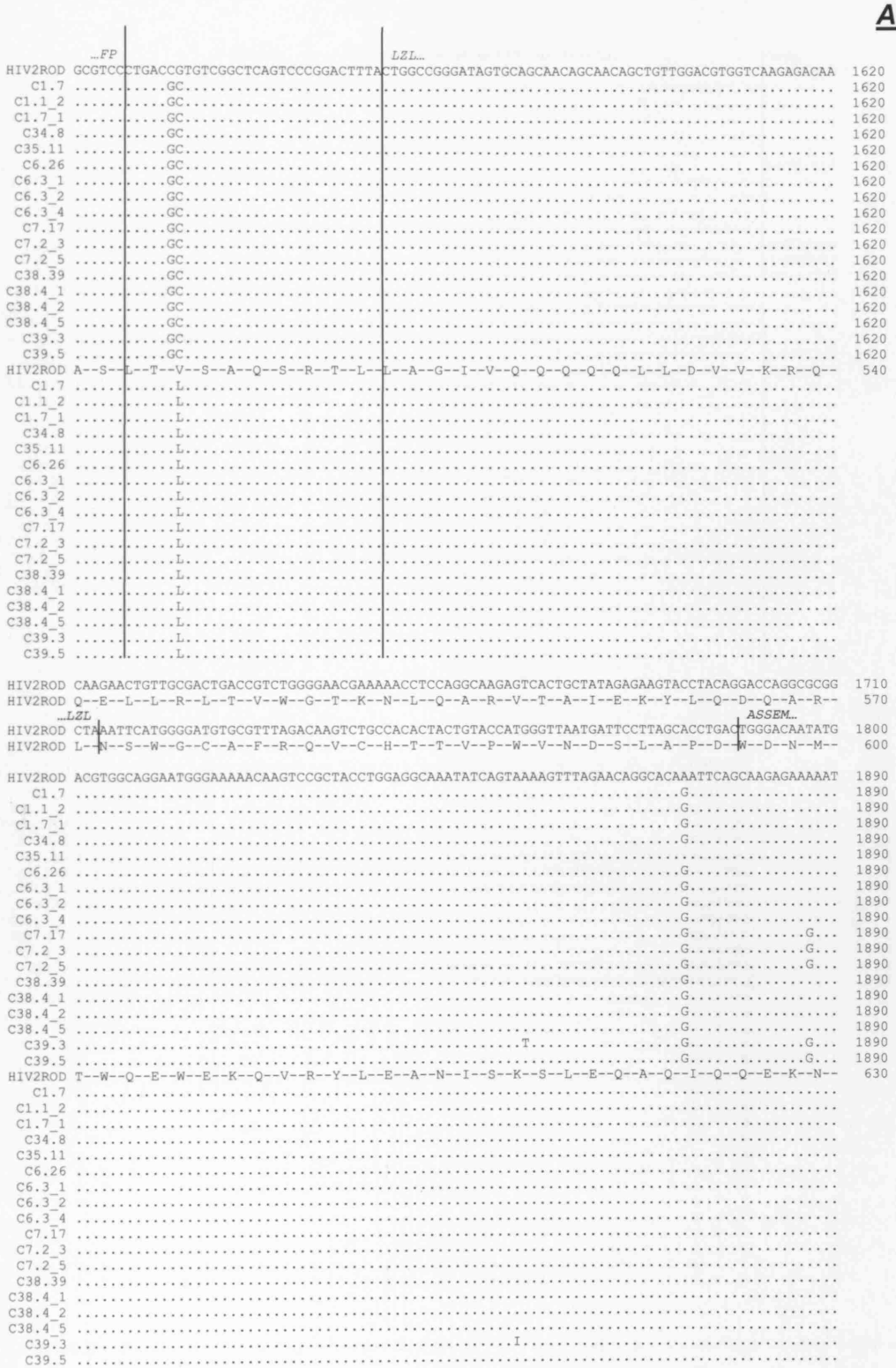
The expression incompetent HIV-2_{ROD} glycoprotein sequences are shown aligned to the prototype HIV-2_{ROD} sequence with (.) indicating identity to the prototype and (-) indicating where gaps were introduced to improve the alignment. The sequences shown here are labelled by their construct ('C' see Table 3.1) and further by their clone name. Only two clones were recovered from multiple experiments regarding HIV-2_{ROD} gp120 construct 2 (Table 3.2 and Figure 3.4). Of these two clones, C2.27 did not express and C2.6 expressed a severely truncated product (results not shown). The

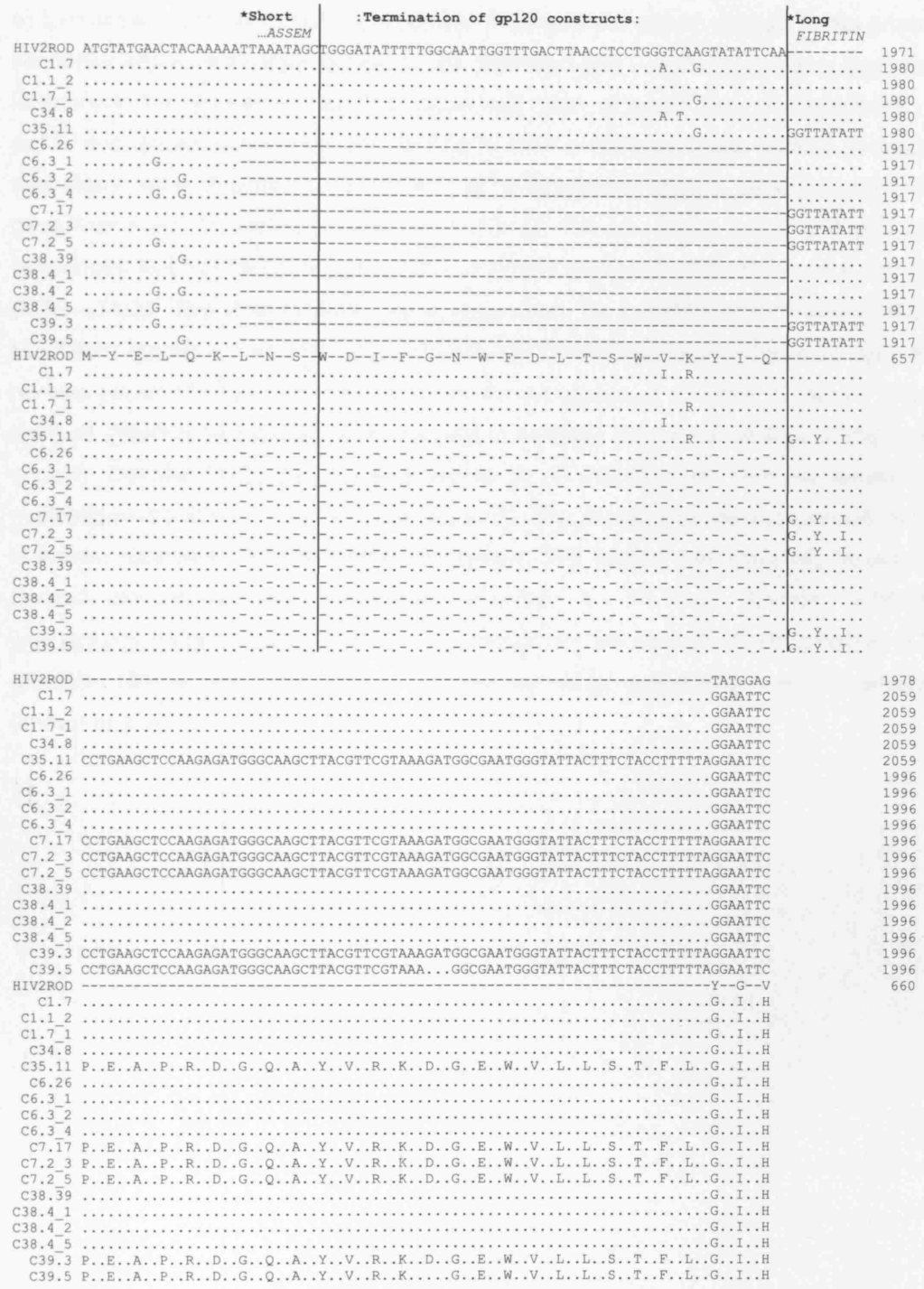
results of sequencing shown here indicate two different disruptions to the *env*-gene. C2.6 has several insertions and deletions which have truncated the gene and caused many amino acid changes in the central region of the gene. Whereas, C2.27 has a thymidine deletion very early in the sequence which leads to a frame shift in the amino acid sequence and an early stop codon. The 3' *Eco*R1 sites (GAATTC), used for cloning are underlined. The figure is annotated as for Appendix Figure 2.


a)
C1…
HIV2ROD TATGTAACTGTTTTCTATGGCGTACCCACGTGGAAAAATGCAACCATTCCCCTCTTTTGTGCAACCAGAAATAGGGATACTTGGGGAACC 90
C1.7 .....G.....A.....A..... 90
C1.1_2 .....G.....A..... 90
C1.7_1 .....G.....A..... 90
C34.8 .....G.....A.....A..... 90
C35.11 .....G.....A.....A..... 90
C6.26 .....G.....A..... 90
C6.3_1 .....G.....A.....A..... 90
C6.3_2 .....G.....A..... 90
C6.3_4 .....G.....A.....A..... 90
C7.17 .....G.....A.....A..... 90
C7.2_3 .....G.....A..... 90
C7.2_5 .....G.....A.....A..... 90
C38.39 .....G.....A.....A..... 90
C38.4_1 .....G.....A.....A..... 90
C38.4_2 .....G.....A..... 90
C38.4_5 .....G.....A.....A..... 90
C39.3 .....G.....A..... 90
C39.5 .....G.....A.....A..... 90
HIV2ROD Y--V--T--V--F--Y--G--V--P--T--W--K--N--A--T--I--P--L--F--C--A--T--R--N--R--D--T--W--G--T-- 30
C1.7 .....I.....N.....
C1.1_2 .....N.....
C1.7_1 .....N.....
C34.8 .....I.....N.....
C35.11 .....I.....N.....
C6.26 .....N.....
C6.3_1 .....I.....N.....
C6.3_2 .....N.....
C6.3_4 .....I.....N.....
C7.17 .....I.....N.....
C7.2_3 .....N.....
C7.2_5 .....I.....N.....
C38.39 .....I.....N.....
C38.4_1 .....I.....N.....
C38.4_2 .....N.....
C38.4_5 .....I.....N.....
C39.3 .....I.....
C39.5 .....I.....N.....
HIV2ROD ATACAGTGCTTGCCTGACAATGATGATTATCAGGAAATAACTTTGAATGTAACAGAGGCTTTTGATGCATGGAATAATACAGTAACAGAA 180
HIV2ROD I--Q--C--L--P--D--N--D--D--Y--Q--E--I--T--L--N--V--T--E--A--F--D--A--W--N--N--T--V--T--E-- 60
…C1 V1…
HIV2ROD CAAGCAATAGAAGATGTCTGGCATCTATTCGAGACATCAATAAAACCATGTGTCAAACTAACACCTTTATGTGTAGCAATGAAATGCAGC 270
HIV2ROD Q--A--I--E--D--V--W--H--L--F--E--T--S--I--K--P--C--V--K--L--T--P--L--C--V--A--M--K--C--S-- 90
HIV2ROD AGCACAGAGAGCAGCACAGGGAACAACACAACCTCAAAGAGCACAAGCACAACCACAACCACACCCACAGACCAGGAGCAAGAGATAAGT 360
HIV2ROD S--T--E--S--S--T--G--N--N--T--T--S--K--S--T--S--T--T--T--T--T--P--T--D--Q--E--Q--E--I--S-- 120
…V1 V2…
HIV2ROD GAGGATACTCCATGCGCACGCGCAGACAACTGCTCAGGATTGGGAGAGGAAGAAACGATCAATTGCCAGTTCAATTATGACAGGATTAGAA 450
C1.7 .....G..... 450
C1.1_2 .....G..... 450
C1.7_1 .....G..... 450
C34.8 .....G..... 450
C35.11 450
C6.26 .....G..... 450
C6.3_1 .....G..... 450
C6.3_2 .....G..... 450
C6.3_4 .....G..... 450
C7.17 .....G..... 450
C7.2_3 .....G..... 450
C7.2_5 .....G..... 450
C38.39 .....G..... 450
C38.4_1 .....G..... 450
C38.4_2 .....G..... 450
C38.4_5 .....G..... 450
C39.3 .....G..... 450
C39.5 .....G..... 450
HIV2ROD E--D--T--P--C--A--R--A--D--N--C--S--G--L--G--E--E--E--T--I--N--C--Q--F--N--M--T--G--L--E-- 150
HIV2ROD AGAGATAAGAAAAAACAGTATAATGAAACATGGTACTCAAAAGATGTGGTTTGTGAGACAAATAATAGCACAAATCAGACCCAGTGTTAC 540
HIV2ROD R--D--K--K--K--Q--Y--N--E--T--W--Y--S--K--D--V--V--C--E--T--N--N--S--T--N--Q--T--Q--C--Y-- 180
…V2 C2…
HIV2ROD ATGAACCATTGCAACACATCAGTCATCACAGAATCATGTGACAAGCACTATTGGGATGCTATAAGGTTTAGATACTGTGCACCACCGGGT 630
HIV2ROD M--N--H--C--N--T--S--V--I--T--E--S--C--D--K--H--Y--W--D--A--I--R--F--R--Y--C--A--P--P--G-- 210
HIV2ROD TATGCCCTATTAAGATGTAATGATACCAATTATTCAGGCTTTGCACCCAACTGTTCTAAAGTAGTAGCTTCTACATGCACCAGGATGATG 720
HIV2ROD Y--A--L--L--R--C--N--D--T--N--Y--S--G--F--A--P--N--C--S--K--V--V--A--S--T--C--T--R--M--M-- 240
HIV2ROD GAAACGCAAACTTCCACATGGTTTGGCTTTAATGGCACTAGAGCAGAGAATAGAACATATATCTATTGGCATGGCAGAGATAATAGAACT 810
HIV2ROD E--T--Q--T--S--T--W--F--G--F--N--G--T--R--A--E--N--R--T--Y--I--Y--W--H--G--R--D--N--R--T-- 270


...C2 V3...
...V3 C3...
...C3 V4...
...V4 C4...
...C4 V5...
...V5 C5...


Amino acid substitutions to prevent gp105/gp15 processing:
E
T
…C5
FP…
HIV2ROD GAGGTGGCAGAACTATACAGATTGGAGTTGGGAGATTATAAATTGGTAGAAATAACACCAATTGGCTTCGCACCTACAAAAGAAAAAAGA 1440
C1.7 1440
C1.1_2 1440
C1.7_1 1440
C34.8 .....TC....G..... 1440
C35.11 .....TC....G..... 1440
C6.26 1440
C6.3_1 1440
C6.3_2 1440
C6.3_4 1440
C7.17 1440
C7.2_3 1440
C7.2_5 1440
C38.39 .....TC....G..... 1440
C38.4_1 .....TC....G..... 1440
C38.4_2 .....TC....G..... 1440
C38.4_5 .....TC....G..... 1440
C39.3 .....CC....G..... 1440
C39.5 .....TC....G..... 1440
HIV2ROD E--V--A--E--L--Y--R--L--E--L--G--D--Y--K--L--V--E--I--T--P--I--G--F--A--P--T--K--E--K--R-- 480
C1.7
C1.1_2
C1.7_1
C34.8 .....S.....E.....
C35.11 .....S.....E.....
C6.26
C6.3_1
C6.3_2
C6.3_4
C7.17
C7.2_3
C7.2_5
C38.39 .....S.....E.....
C38.4_1 .....S.....E.....
C38.4_2 .....S.....E.....
C38.4_5 .....S.....E.....
C39.3 .....P.....E.....
C39.5 .....S.....E.....
HIV2ROD TACTCCTCTGCTCACGGGAGACATACAAGAGGTGTGTTCGTGCTAGGGTTCTTGGGTTTTCTCGCAACAGCAGGTTCTGCAATGGGCGCG 1530
C1.7 1530
C1.1_2 1530
C1.7_1 1530
C34.8 .....CA.T....A.....C.....T..... 1530
C35.11 .....CA.T....A.....C..... 1530
C6.26 1530
C6.3_1 1530
C6.3_2 1530
C6.3_4 1530
C7.17 1530
C7.2_3 1530
C7.2_5 1530
C38.39 .....A.T....A.....C..... 1530
C38.4_1 .....A.T....A.....C..... 1530
C38.4_2 .....A.T....A.....C..... 1530
C38.4_5 .....A.T....A.....C..... 1530
C39.3 .....CA.T....A.....C..... 1530
C39.5 .....CA.T.....C.....T..... 1530
HIV2ROD Y--S--S--A--H--G--R--H--T--R--G--V--F--V--L--G--F--L--G--F--L--A--T--A--G--S--A--M--G--A-- 510
C1.7
C1.1_2
C1.7_1
C34.8 .....P..V.....N.....T..
C35.11 .....P..V.....N.....T..
C6.26
C6.3_1
C6.3_2
C6.3_4
C7.17
C7.2_3
C7.2_5
C38.39 .....Q..V.....N.....T..
C38.4_1 .....Q..V.....N.....T..
C38.4_2 .....Q..V.....N.....T..
C38.4_5 .....Q..V.....N.....T..
C39.3 .....P..V.....N.....T..
C39.5 .....P..V.....T..


…FP
LZL…
HIV2ROD GCGTCCCTGACCGTGTCGGCTCAGTCCCGGACTTTACTGGCCGGGATAGTGCAGCAACAGCAACAGCTGTTGGACGTGGTCAAGAGACAA 1620
HIV2ROD A--S--L--T--V--S--A--Q--S--R--T--L--L--A--G--I--V--Q--Q--Q--Q--Q--Q--L--L--D--V--V--K--R--Q-- 540
HIV2ROD CAAGAACTGTTGCGACTGACCGTCTGGGGAACGAAAAACCTCCAGGCAAGAGTCACTGCTATAGAGAAGTACCTACAGGACCAGGCGCGG 1710
HIV2ROD Q--E--L--L--R--L--T--V--W--G--T--K--N--L--Q--A--R--V--T--A--I--E--K--Y--L--Q--D--Q--A--R-- 570
…LZL
ASSEM…
HIV2ROD CTAAATTCATGGGGATGTGCGTTTAGACAAGTCTGCCACACTACTGTACCATGGGTTAATGATTCCTTAGCACCTGACTGGGACAATATG 1800
HIV2ROD L--N--S--W--G--C--A--F--R--Q--V--C--H--T--T--V--P--W--V--N--D--S--L--A--P--D--W--D--N--M-- 600
HIV2ROD ACGTGGCAGGAATGGGAAAAACAAGTCCGCTACCTGGAGGCAAATATCAGTAAAAGTTTAGAACAGGCACAAATTCAGCAAGAGAAAAAT 1890
HIV2ROD T--W--Q--E--W--E--K--Q--V--R--Y--L--E--A--N--I--S--K--S--L--E--Q--A--Q--I--Q--Q--E--K--N-- 630



b)

HIV2ROD G--F--A--P--T--K--E--K--R--Y--S--S--A--H--G--R--H--T--R--G--V--F--V--L--G--F–

HIV2ROD GGCTTCGCACCTACAAAAGAAAAAAGATACTCCTCTGCTCACGGGAGACATACAAGAGGTGTGTTCGTGCTAGGGTTC

H2REMF GGCTTCGCACCTACAYCAGAAGAAAGATACTCCTCTGCTCMAGTGAGAMATACAACAGGTGTGTTYGTGCTAGGGTTC

POSS G--F--A--P--T--S--E--E--R--Y--S--S--A--Q--V--R--H--T--T--G--V--F--V--L--G--F--

POSS G--F--A--P--T--P--E--E--R--Y--S--S--A--P--V--R--N--T--T--G--V--F--V--L--G--F--

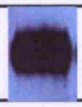
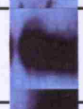
Appendix Figure 6: HIV-2_{ROD} gp120 sequences of expression competent pEE14tPA-*env* clones selected for constitutive protein expression studies

a) Expression competent HIV-2_{ROD} gp120 constructs are shown aligned to the prototype HIV-2_{ROD} sequence with (.) indicating identity to the prototype and (-) indicating where gaps were introduced to improve the alignment. The tPA signal has been removed from this alignment. The HIV-2_{ROD} gp120 sequences shown here are labelled by their construct ('C' see Table 3.2) and then further by their clone name. The figure is annotated as for Appendix Figure 3. Initial screen of HIV-2_{ROD} clones gave four intact sequences, these were C34.8, C35.11, C39.3 and C39.5 and many produced sequences that contained a stop codon at amino acid position 318 due to a Trp codon mutation (TGG→TAG). This was repaired using sequences C1.7, C6.26, C7.17 and C38.39 and primers H2CORF (position 941-968 CCCAGACAAGCAT-GGTGCTGGTTCAAAG) and H2CORR (the reverse primer of H2CORF). This enabled the production of a further 10 clones (see above) which were all shown to be expression competent in transient expression assays in 293T cells (results not shown). Despite using the Pfu-polymerase in all PCRs, there is some evidence for sporadic nucleotide substitution in some of the clones. b) The processing site mutagenesis primer (H2REMF) is shown relative to the HIV-2ROD sequence. The primer was designed based on the patient-derived env-clones and sequences available in the Los Alamos database (http://hiv-web.lanl.gov/content/index). Hence, in addition to the desired K→E (position 479) and R→T (position 490) substitutions (shown in red), four other amino acid positions showed substitution (shown in green).

Record	Specimen	Age*	Sex	Proviral Copy No.	No clones
>60					
185	B1222	66	F	5.3	2
213	B1255	66	F	6.8	2
188	B1229	70	F	9.5	2
159	B1196	66	F	14.1	2
223	B1265	66	F	60.2	7
220	B1262	70	F	84.7	2
057	B1065	70	F	98.2	2
150	B1187	74	F	213.2	3
114	B1123	66	F	280.5	2
232	B1276	82	F	848.5	5
146	B1183	73	M	8667.5	4
<40					
135	B1172	38	F	42.6	2
170	B1207	38	F	65.5	6
123	B1160	18	F	87.1	4
076	B1084	15	F	129.4	2
242	B1286	29	M	174.4	3
258	B1302	38	F	322.1	2
249	B1293	25	F	323.4	3
101	B1109	37	M	764.0	2
195	B1236	38	F	1110.9	2
066	B1074	38	F	4705.9	3
112	B1121	33	M	4947.2	4

Appendix Table 1: Summary of Caio Samples for which *env*-gene sequencing completed

The information in the first five columns was supplied with the peripheral blood mononuclear cell (PBMC) samples. Patient record numbers are given for the two age-grouped patient cohorts and the specimen number relates to the particular sampling performed in 1991. * Age at time of sampling. The proviral copy number/100,000 PBMC was determined by Dr Koya Ariyoshi. The number of expression competent *env*-gene clones sequenced for each patient sample is shown, 66 in total.

Antibody[a]	Epitope[b]	Concentration mg/ml[c]	K_D[d]	Native gel[e]	Immunoprecipitation[e]	Vivaspn Column[e]
3083	*44.5j* – C1 DDYQEITLNVTE	0.640	8.58×10^{-9}			
3084	*25.8c* – V1 SEDTPCARA	0.549	1.47×10^{-8}			
3085	*44.5k* – V2 GEEETINCQ	0.398	2.17×10^{-8}			
3086	*44.2g* – V2 FNMTGL	0.835	1.91×10^{-8}			N/D
3087	*28.3e* CD	0.622	2.77×10^{-7}			
3088	*28.8e* CD	0.346	7.25×10^{-8}			
3089	*25.3f* CD	0.568	2.57×10^{-6}			
3090	*32.2f* – V3 LMSGHVFHSHYQ	0.429	5.47×10^{-6}			
3091	*32.7g* – V3 SGHVFHSHYQ	0.353	6.26×10^{-8}			
3032	*PQ41* C2	2.033	8.14×10^{-7}			
3030	*PQ2G12* C1	5.492	5.28×10^{-8}			

Appendix Table 2: Detailed results of Immunoaffinity Measurements

See Table 3.3 for legend. [e] For western blot results detection of HIV-2_{ROD} gp120 is shown. For Native gel western blots were probed with relevant antibody at dilution stated in Section 2.2.4 and developed for 2min. IP and Vivaspin columns western blots were probed with ARP 3030 at a dilution of 1:10 (TCSN) followed by Goat anti-Mouse IgG HRP at a dilution of 1:5000 and then developed for 2min and 20min respectively.

	Mechanism to Enhance Protein Association				
	Salt	PEG	Organic		
Reagent	After the activity Coefficient of water	Increase Molecular Crowding	Reduce the Solvent Dielectric	pH	Additive
1	2M Am_2SO_4		2% PEG400	5.5	
2	2M Am_2SO_4		10% Glycerol	6.5	0.1M $MgSO_4$
3	2M Am_2SO_4		1% MPD	7.5	
4	2M Am_2SO_4		5% PEG400	8.5	0.1M $MgSO_4$
5	4M NaCl		2% PEG400	5.5	0.1M $MgCl_2$
6	3M NaCl		5% MPD	6.5	0.1M $CaCl_2$
7	4M NaCl		5% Isopropanol	7.5	
8	2.5M $NaKPO_4$		5% Isopropanol	5.5	
9	2M $NaKPO_4$		2% PEG400	6.5	
10	2.5M $NaKPO_4$		20% Glycerol	7.5	
11	1M $NaKPO_4$		8% MPD	8.5	
12	2M AmCitrate		1% MPD	4.5	
13	2M AmCitrate		5% Isopropanol	6.5	
14	2M AmCitrate		5% PEG400	7.5	
15	2M Li_2SO_4		5% Isopropanol	4.5	0.1M $MgSO_4$
16	2M Li_2SO_4		5% PEG400	5.5	0.1M $MgSO_4$
17	2M Li_2SO_4		8% MPD	6.5	
18	2M Li_2SO_4		2% PEG400	8.5	
19	1M Li_2SO_4		15% MPD	4.5	0.1M $MgSO_4$
20	0.75M AmCitrate		25% MPD	5.5	
21	1.5M Am_2SO_4		12% Isopropanol	6.5	
22	1.3M NaCl		30% Isopropanol	6.5	0.1M $CaCl_2$
23	4M NaCl		10% PEG400	7.5	
24	0.8M $NaKPO_4$		20% PEG400	7.5	
25	1M AmCitrate		15% Isopropanol	8.5	
26	2M NaFormate	2.5% PEG3350	15% Isopropanol	8.5	
27		***25% PEG1500***	***30% MPD***	***4.5***	
28		*15% PEG8000*	*30% MPD*	*5.5*	*0.1M $CaCl_2$*
29		*10% PEG3350*	*30% MPD*	*6.5*	*0.2M Am_2SO_4*
30		4% PEG1500	30% MPD	7.5	
31		8% PEG8000	30% MPD	8.5	0.5M NaCl
32		4% PEG3350	30% Isopropanol	4.5	0.1M $CaCl_2$
33		10% PEG1500	30% Isopropanol	5.5	0.2M Li_2SO_4
34		15% PEG8000	40% Isopropanol	6.5	
35		15% PEG3350	20% Isopropanol	7.5	0.2M AmCitrate
36		30% PEG3350	30% Isopropanol	8.5	
37		*20% PEG8000*	*40% PEG400*	*4.5*	
38		5% PEG3350	40% PEG400	5.5	
39		15% PEG1000	40% PEG400	6.5	0.15M $NaKPO_4$
40		*8% PEG8000*	*40% PEG400*	*7.5*	
41		***20% PEG3350***	***25% PEG400***	***8.5***	***0.1M $MgCl_2$***
42		*30% PEG1500*	*3% MPD*	*5.5*	*0.2M $MgSO_4$*
43		*30% PEG1500*	*10% Isopropanol*	*6.5*	*0.1M $CaCl_2$*
44		*30% PEG1500*	*20% PEG400*	*7.5*	
45		*30% PEG1500*	*8% MPD*	*8.5*	
46		25% PEG3350	15% Isopropanol	4.5	0.2M AmCitrate
47		*25% PEG3350*	*5% PEG400*	*5.5*	
48		***25% PEG3350***	***15% MPD***	***6.5***	***0.2M Li_2SO_4***
49		***25% PEG3350***	***4% Isopropanol***	***7.5***	***0.1M $CaCl_2$***
50		*20% PEG8000*	*10% PEG400*	*5.5*	*0.5M NaCl*
51		20% PEG8000	3% MPD	6.5	
52		*20% PEG8000*	*10% Isopropanol*	*7.5*	*0.2M Am_2SO_4*
53		20% PEG8000	20% PEG400	8.5	0.1M $MgCl_2$
54	3M NaFormate	25% PEG3350		4.5	0.1M $CaCl_2$
55	0.75M Am_2SO_4	7.5% PEG3350	5% Isopropanol	4.5	
56	1M AmCitrate	1% PEG4000		5.5	
57	2.5M NaCl	12% PEG1500	1.5% MPD	5.5	
58	3M NaCl	20% PEG3350		6.5	0.1M $MgCl_2$
59	3M NaFormate	4% PEG8000		6.5	
60	1M $NaKPO_4$	0.5% PEG4000		7.5	
61	1.4M $NaKPO_4$	10% PEG3350		7.5	
62	0.8M AmCitrate	2% PEG8000		8.5	
63	2M NaCl	5% PEG4000		8.5	
64	0.5M AmCitrate	15% PEG8000		8.5	

Appendix Table 3: Precipitant Synergy Reagent Formulation

The mother liquors used in the PS Screen are shown (Majeed et al., 2003). The reagents indicated in *italics* are those that produced medium to heavy precipitates in the initial screen and of these, those indicated in bold are the conditions I chose to optimise (Table 3.5 and Figure 3.37). MPD = (±) -2-Methyl-2,4-pentanediol, PEG = Polyethylene Glycol.

A: Classics Screen (Nextal Biotechnologies)

Sample: 39.5 8 Sample concentration: 1.3mg/ml

Sample buffer: 1M NaCl 50mM HEPES pH6.5 Date: 18/11/05

Reservoir volume: 100µl Temperature: 18°C

Drop volume: Total 0.3µl Sample 0.2µl Reservoir 0.1µl

clear	clear	clear	clear	clear	clear	hair	clear	ps	dark ps	clear	clear
clear	clear	clear	clear	clear	clear	clear	ps	ps and needle	mass	clear	clear
clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear
clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear
N/A	ps	clear	clear	clear	clear	ring + ps	clear	clear	clear	clear	clear
clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	mass	clear
ps	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear
clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear	clear

B: Stura Footprint and Macrasol screens (Molecular Dimensions Ltd)

Sample: 39.5 8 Sample concentration: 1.3mg/ml

Sample buffer: 1M NaCl 50mM HEPES pH 6.5 Date: 18/11/05

Reservoir volume: 100µl Temperature: 18°C

Drop volume: Total 0.3µl Sample 0.2µl Reservoir 0.1µl

ps	ps	ps	clear	ps	ps	ps	clear	mass	clear	clear	clear
ps	ps	ps	clear	ps	clear	ps	ps	clear	clear	ps	ps
ps	ps	ps	l ps	ps	ps	ps	clear	clear	clear	l ppt	l ppt
ps	ps	ps	ps	clear	ps	ps	m ppt	clear	clear	clear	l ppt + mass
ps	ps	ps	ps	ps	ps	ps	clear	clear	clear	clear	l ppt
ps	ps	ps	ps	ps	clear	clear	clear	ring	ring	Ring + l ppt	Ring
ps	ps	ps	ps	ps	clear	ps	ps and l ppt	ps	clear	clear	l ppt
ps	ps	ps	ps	ps	clear	l ps	clear	ps	clear	clear	m ppt

Legend

(l) Ps - (light) phase separation (Figure 3.37a)

l/m ppt - light/medium precipitate (Figure 3.37b)

Clear - no reaction in drop (Figure 3.37c)

ring - possibly skin/large ps drop within the drop

mass - not ps or ppt but something present in the drop

Appendix Table 4: Examples of results from robotic screens

Shown here are the results from the robotics screens of conditions A: Classics Screen (Nextal Biotechnologies) and B: Stura Footprint and Macrosol screens (Molecular Dimensions Ltd) which were set up in 96 well plates. These are the results at 3 months after setting up the drops. See legend above for codes.

Appendix Method 1: Adsorbing Antibodies using Aldehyde Activated Porus Silica

Materials

Aldehyde activated porus silica (Matrix) (Clifmar Associates 020991)

Cells to be used for adsorbing (293T cells grown in 10% FCS DMEM)

Polyclonal or Monoclonal Antibody to be clarified

PBS

1% Triton x 100/PBS

1M NaCl

0.1M Glycine

Method

Day 1

- Remove medium from a small flask ($25cm^2$) of cells and add 5ml of trypsin.
- Incubate at 37°C for 2min.
- Check under the microscope that cells have rounded and suspended.
- Give flask a shake, then add 7ml of growth medium to inactivate the trypsin.
- Put medium in a 15ml falcon tube.
- Centrifuge at 0.1 x g for 5min at 10°C.
- Remove supernatant and resuspend in 5ml of growth medium.
- Centrifuge again after splitting into 2ml and 3ml.
- Remove supernatant.

- Resuspend the 2ml of whole cells in 50µl of 10% FCS medium.
- To that add 100µl of antiserum (Polyclonal NIH 1410).
- Incubate at 4°C overnight on rollers (A).

- Resuspend the 3ml of cells in 90µl of 1 X Triton mix.
- Leave at room temperature for 10-15min to lyse. Then keep the lysate at 4°C.

- Weigh out 0.09g of matrix and mix with 260µl of PBS.
- Incubate for 4h at room temperature on a mixer.
- Add lysate to matrix and incubate overnight at room temperature (B).

Day 2

- Wash matrix (B) with 2 x 1ml of PBS, then 2 x 1ml 1M NaCl, then 2 x 1ml 0.1M Glycine HCl pH2.5 then finally 2 x 0.75ml PBS. Between the washes centrifuge the sample (15.7 x g/2min) and discard supernatants.
- Centrifuge the antibody-whole cell mix (A) at 15.7 x g for 5min at 4°C, discard the pellet and keep the supernatant.
- Add the supernatant (A) to the matrix (B) and incubate for 4h at room temperature (rotating).
- Finally centrifuge the mixture at 15.7 x g for 5min and freeze aliquots of the supernatant (1 x 100µl and 1 x 75µl).

Appendix Method 2: Preparation of a 10% Polyacrylamide gel

Materials

Resolving Gel	ml/gel
DDW	– 1.98
30% acrylamide mix	– 1.67
Protogel Buffer	– 1.3
10% ammonium persulphate	– 0.05
TEMED	– 0.005
	~5ml

Stacking Gel	ml/gel
DDW	– 1.21
30% acrylamide mix	– 0.266
0.5M Tris (pH6.8)	– 0.5
10% SDS	– 0.02
10% ammonium persulphate	– 0.02
TEMED	– 0.004
	~2ml

For both the resolving and the stacking gel add the TEMED last as this causes the gel to set.

Appendix Method 3: Preparation of 5-15% Gradient SDS PAGE

Materials

	5%	15%
DDW/ml	10.7	4.4
1.5M Tris pH8.8/ml	4.75	4.75
30% Acrylamide/ml	3.2	9.5
10% SDS/µl	95	95
10% Ammonium persulphate/µl	190	190
TEMED/µl	19	19

This produces eight gels. A multi casting chamber (BIO-RAD, Cat. No. 165-4110) is required in which eight front and back plates are placed separated by a plastic sheet. Each glass plate is cleaned with 70% ethanol before placing in the casting tank. The solutions were made up minus TEMED and placed in a gradient former (BIO-RAD, Cat. No. 165-4120) (15% in the outside funnel and 5% closest to casting chamber). Before the mixes are allowed to flow TEMED is added and the 5% is mixed as it enters the casting chamber.

Appendix Method 4: Preparation of 4-12% Native PAGE

Materials

	4%	12%
DDW/ml	11.4	6.3
1.5M Tris pH8.8/ml	4.75	4.75
30% Acrylamide/ml	2.5	7.6
10% Ammonium persulphate/µl	190	190
TEMED/µl	19	19

This produces eight gels as described for Appendix Method 3.

Chapter 6

References

6 References

Aaby, P., Poulsen, A. G., Larsen, O., Christiansen, C. B., Jensen, H., Melbye, M., Naucler, A., and Dias, F. (1997). Does HIV-2 protect against HIV-1 infection? *AIDS* **11**(7), 939-40.

Abada, P., Noble, B., and Cannon, P. M. (2005). Functional Domains within the Human Immunodeficiency Virus Type 2 Envelope Protein Required To Enhance Virus Production. *J Virol* **79**(6), 3627-38.

Abbott, R. C., A, N. D.-S., Diouf, A., T, N. D., Ba, D., Tall, N., N, M. B., Kebe, F., Diadhiou, F., Essex, M. E., and et al. (1994). Risk factors for HIV-1 and HIV-2 infection in pregnant women in Dakar, Senegal. *J Acquir Immune Defic Syndr* **7**(7), 711-7.

Adjorlolo-Johnson, G., De Cock, K. M., Ekpini, E., Vetter, K. M., Sibailly, T., Brattegaard, K., Yavo, D., Doorly, R., Whitaker, J. P., Kestens, L., and et al. (1994). Prospective comparison of mother-to-child transmission of HIV-1 and HIV-2 in Abidjan, Ivory Coast. *Jama* **272**(6), 462-6.

Affranchino, J. L., and Gonzalez, S. A. (2006). Mutations at the C-terminus of the simian immunodeficiency virus envelope glycoprotein affect gp120-gp41 stability on virions. *Virology* **347**(1), 217-25.

Ahmad, N., Baroudy, B. M., Baker, R. C., and Chappey, C. (1995). Genetic analysis of human immunodeficiency virus type 1 envelope V3 region isolates from mothers and infants after perinatal transmission. *J Virol* **69**(2), 1001-12.

Akahata, W., Yang, Z. Y., and Nabel, G. J. (2005). Comparative immunogenicity of human immunodeficiency virus particles and corresponding polypeptides in a DNA vaccine. *J Virol* **79**(1), 626-31.

Akimoto, H., Kaneko, H., Sekigawa, I., Hashimoto, H., Kaneko, Y., and Yamamoto, N. (1998). Binding of HIV-2 envelope glycoprotein to CD8 molecules and related chemokine production. *Immunology* **95**(2), 214-8.

Alabi, A. S., Jaffar, S., Ariyoshi, K., Blanchard, T., Schim Van Der Loeff, M., Awasana, A. A., Corrah, T., Sabally, S., Sarge-Njie, R., Cham-Jallow, F., Jaye, A., Berry, N., and Whittle, H. (2003). Plasma viral load, CD4 cell percentage, HLA and survival of HIV-1, HIV-2, and dually infected Gambian patients. *Aids* **17**(10), 1513-1520.

Alimonti, J. B., Ball, T. B., and Fowke, K. R. (2003). Mechanisms of CD4(+) T lymphocyte cell death in human immunodeficiency virus infection and AIDS. *J Gen Virol* **84**(Pt 7), 1649-61.

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., and Berger, E. A. (1996). CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**(5270), 1955-8.

Allan, J. S., Coligan, J. E., Barin, F., McLane, M. F., Sodroski, J. G., Rosen, C. A., Haseltine, W. A., Lee, T. H., and Essex, M. (1985). Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. *Science* **228**(4703), 1091-4.

Allaway, G. P., Davis-Bruno, K. L., Beaudry, G. A., Garcia, E. B., Wong, E. L., Ryder, A. M., Hasel, K. W., Gauduin, M. C., Koup, R. A., McDougal, J. S., and et al. (1995). Expression and characterization of CD4-IgG2, a novel heterotetramer that neutralizes primary HIV type 1 isolates. *AIDS Res Hum Retroviruses* **11**(5), 533-9.

Allen, T. M., Vogel, T. U., Fuller, D. H., Mothe, B. R., Steffen, S., Boyson, J. E., Shipley, T., Fuller, J., Hanke, T., Sette, A., Altman, J. D., Moss, B., McMichael, A. J., and Watkins, D. I. (2000). Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen. *J Immunol* **164**(9), 4968-78.

Almond, N., Jenkins, A., Heath, A. B., and Kitchin, P. (1993). Sequence variation in the env gene of simian immunodeficiency virus recovered from immunized macaques is predominantly in the V1 region. *J Gen Virol* **74 (Pt 5)**, 865-71.

Amara, A., Vidy, A., Boulla, G., Mollier, K., Garcia-Perez, J., Alcami, J., Blanpain, C., Parmentier, M., Virelizier, J. L., Charneau, P., and Arenzana-Seisdedos, F. (2003). G protein-dependent CCR5 signaling is not required for efficient infection of primary T lymphocytes and macrophages by R5 human immunodeficiency virus type 1 isolates. *J Virol* **77**(4), 2550-8.

References

Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Herndon, J. G., Wyatt, L. S., Candido, M. A., Kozyr, N. L., Earl, P. L., Smith, J. M., Ma, H. L., Grimm, B. D., Hulsey, M. L., Miller, J., McClure, H. M., McNicholl, J. M., Moss, B., and Robinson, H. L. (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292**(5514), 69-74.

Ancelle, R., Bletry, O., Baglin, A. C., Brun-Vezinet, F., Rey, M. A., and Godeau, P. (1987). Long incubation period for HIV-2 infection. *Lancet* **1**(8534), 688-9.

Anderson, J. L., and Hope, T. J. (2004). HIV accessory proteins and surviving the host cell. *Curr HIV/AIDS Rep* **1**(1), 47-53.

Anderson, R. W., Ascher, M. S., and Sheppard, H. W. (1998). Direct HIV cytopathicity cannot account for CD4 decline in AIDS in the presence of homeostasis: a worst-case dynamic analysis. *J Acquir Immune Defic Syndr Hum Retrovirol* **17**(3), 245-52.

Andersson, S., Makitalo, B., Thorstensson, R., Franchini, G., Tartaglia, J., Limbach, K., Paoletti, E., Putkonen, P., and Biberfeld, G. (1996). Immunogenicity and protective efficacy of a human immunodeficiency virus type 2 recombinant canarypox (ALVAC) vaccine candidate in cynomolgus monkeys. *J Infect Dis* **174**(5), 977-85.

Andersson, S., Norrgren, H., da Silva, Z., Biague, A., Bamba, S., Kwok, S., Christopherson, C., Biberfeld, G., and Albert, J. (2000). Plasma viral load in HIV-1 and HIV-2 singly and dually infected individuals in Guinea-Bissau, West Africa: significantly lower plasma virus set point in HIV-2 infection than in HIV-1 infection. *Arch Intern Med* **160**(21), 3286-93.

Andiman, W. A., Simpson, B. J., Olson, B., Dember, L., Silva, T. J., and Miller, G. (1990). Rate of transmission of human immunodeficiency virus type 1 infection from mother to child and short-term outcome of neonatal infection. Results of a prospective cohort study. *Am J Dis Child* **144**(7), 758-66.

Apetrei, C., Robertson, D., and Marx, P. A. (2004). The history of SIVs and AIDS: Epidemiology, Phylogeny and biology of isolates from naturally SIV infected non-human primates (NHP) in Africa. *Frontiers in Bioscience* **9**, 225-254.

Arakaki, R., Tamamura, H., Premanathan, M., Kanbara, K., Ramanan, S., Mochizuki, K., Baba, M., Fujii, N., and Nakashima, H. (1999). T134, a small-molecule CXCR4 inhibitor, has no cross-drug resistance with AMD3100, a CXCR4 antagonist with a different structure. *J Virol* **73**(2), 1719-23.

Arien, K. K., Abraha, A., Quinones-Mateu, M. E., Kestens, L., Vanham, G., and Arts, E. J. (2005). The replicative fitness of primary human immunodeficiency virus type 1 (HIV-1) group M, HIV-1 group O, and HIV-2 isolates. *J Virol* **79**(14), 8979-90.

Ariyoshi, K., Schim van der Loeff, M., Cook, P., Whitby, D., Corrah, T., Jaffar, S., Cham, F., Sabally, S., O'Donovan, D., Weiss, R. A., Schulz, T. F., and Whittle, H. (1998). Kaposi's sarcoma in the Gambia, West Africa is less frequent in human immunodeficiency virus type 2 than in human immunodeficiency virus type 1 infection despite a high prevalence of human herpesvirus 8. *J Hum Virol* **1**(3), 193-9.

Ariyoshi, K., Schim van der Loeff, M., Sabally, S., Cham, F., Corrah, T., and Whittle, H. (1997). Does HIV-2 infection provide cross-protection against HIV-1 infection? *AIDS* **11**(8), 1053-4.

Arthos, J., Deen, K. C., Chaikin, M. A., Fornwald, J. A., Sathe, G., Sattentau, Q. J., Clapham, P. R., Weiss, R. A., McDougal, J. S., Pietropaolo, C., and et al. (1989). Identification of the residues in human CD4 critical for the binding of HIV. *Cell* **57**(3), 469-81.

Arthos, J., Rubbert, A., Rabin, R. L., Cicala, C., Machado, E., Wildt, K., Hanbach, M., Steenbeke, T. D., Swofford, R., Farber, J. M., and Fauci, A. S. (2000). CCR5 signal transduction in macrophages by human immunodeficiency virus and simian immunodeficiency virus envelopes. *J Virol* **74**(14), 6418-24.

Arthur, L. O., Bess, J. W., Jr., Sowder, R. C., 2nd, Benveniste, R. E., Mann, D. L., Chermann, J. C., and Henderson, L. E. (1992). Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. *Science* **258**(5090), 1935-8.

Ashkenazi, A., Presta, L. G., Marsters, S. A., Camerato, T. R., Rosenthal, K. A., Fendly, B. M., and Capon, D. J. (1990). Mapping the CD4 binding site for human immunodeficiency virus by alanine-scanning mutagenesis. *Proc Natl Acad Sci U S A* **87**(18), 7150-4.

Ashkenazi, A., Smith, D. H., Marsters, S. A., Riddle, L., Gregory, T. J., Ho, D. D., and Capon, D. J. (1991). Resistance of primary isolates of human immunodeficiency virus type 1 to soluble CD4 is independent of CD4-rgp120 binding affinity. *Proc Natl Acad Sci U S A* **88**(16), 7056-60.

References

Ayouba, A., Souquieres, S., Njinku, B., Martin, P. M., Muller-Trutwin, M. C., Roques, P., Barre-Sinoussi, F., Mauclere, P., Simon, F., and Nerrienet, E. (2000). HIV-1 group N among HIV-1-seropositive individuals in Cameroon. *AIDS* **14**(16), 2623-5.

Azad, A. A. (2000). Could Nef and Vpr proteins contribute to disease progression by promoting depletion of bystander cells and prolonged survival of HIV-infected cells? *Biochem Biophys Res Commun* **267**(3), 677-85.

Azevedo-Pereira, J. M., Santos-Costa, Q., and Moniz-Pereira, J. (2005). HIV-2 infection and chemokine receptors usage - clues to reduced virulence of HIV-2. *Curr HIV Res* **3**(1), 3-16.

Baba, M., Nishimura, O., Kanzaki, N., Okamoto, M., Sawada, H., Iizawa, Y., Shiraishi, M., Aramaki, Y., Okonogi, K., Ogawa, Y., Meguro, K., and Fujino, M. (1999). A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *Proc Natl Acad Sci U S A* **96**(10), 5698-703.

Babu, P. G., Saraswathi, N. K., Devapriya, F., and John, T. J. (1993). The detection of HIV-2 infection in southern India. *Indian J Med Res* **97**, 49-52.

Back, N. K., Smit, L., De Jong, J. J., Keulen, W., Schutten, M., Goudsmit, J., and Tersmette, M. (1994). An N-glycan within the human immunodeficiency virus type 1 gp120 V3 loop affects virus neutralization. *Virology* **199**(2), 431-8.

Bahraoui, E., Benjouad, A., Guetard, D., Kolbe, H., Gluckman, J. C., and Montagnier, L. (1992). Study of the interaction of HIV-1 and HIV-2 envelope glycoproteins with the CD4 receptor and role of N-glycans. *AIDS Res Hum Retroviruses* **8**(5), 565-73.

Balliet, J. W., Kolson, D. L., Eiger, G., Kim, F. M., McGann, K. A., Srinivasan, A., and Collman, R. (1994). Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes vpr, vpu, and nef: mutational analysis of a primary HIV-1 isolate. *Virology* **200**(2), 623-31.

Balzarini, J., Hatse, S., Vermeire, K., Princen, K., Aquaro, S., Perno, C. F., De Clercq, E., Egberink, H., Vanden Mooter, G., Peumans, W., Van Damme, E., and Schols, D. (2004). Mannose-specific plant lectins from the Amaryllidaceae family qualify as efficient microbicides for prevention of human immunodeficiency virus infection. *Antimicrob Agents Chemother* **48**(10), 3858-70.

Balzarini, J., Kruining, J., Wedgwood, O., Pannecouque, C., Aquaro, S., Perno, C. F., Naesens, L., Witvrouw, M., Heijtink, R., De Clercq, E., and McGuigan, C. (1997). Conversion of 2',3'-dideoxyadenosine (ddA) and 2',3'-didehydro-2',3'-dideoxyadenosine (d4A) to their corresponding aryloxyphosphoramidate derivatives markedly potentiates their activity against human immunodeficiency virus and hepatitis B virus. *FEBS Lett* **410**(2-3), 324-8.

Balzarini, J., Wedgwood, O., Kruining, J., Pelemans, H., Heijtink, R., De Clercq, E., and McGuigan, C. (1996). Anti-HIV and anti-HBV activity and resistance profile of 2',3'-dideoxy-3'-thiacytidine (3TC) and its arylphosphoramidate derivative CF 1109. *Biochem Biophys Res Commun* **225**(2), 363-9.

Banda, N. K., Bernier, J., Kurahara, D. K., Kurrle, R., Haigwood, N., Sekaly, R. P., and Finkel, T. H. (1992). Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. *J Exp Med* **176**(4), 1099-106.

Barouch, D. H., and Letvin, N. L. (2000). DNA vaccination for HIV-1 and SIV. *Intervirology* **43**(4-6), 282-7.

Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X., Krivulka, G. R., Beaudry, K., Lifton, M. A., Nickerson, C. E., Trigona, W. L., Punt, K., Freed, D. C., Guan, L., Dubey, S., Casimiro, D., Simon, A., Davies, M. E., Chastain, M., Strom, T. B., Gelman, R. S., Montefiori, D. C., Lewis, M. G., Emini, E. A., Shiver, J. W., and Letvin, N. L. (2000). Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290**(5491), 486-92.

Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W., and Montagnier, L. (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**(4599), 868-71.

Barreto, J., Liljestrand, J., Palha de Sousa, C., Bergstrom, S., Bottiger, B., Biberfeld, G., and De la Cruz, F. (1993). HIV-1 and HIV-2 antibodies in pregnant women in the City of Maputo, Mozambique. A comparative study between 1982/1983 and 1990. *Scand J Infect Dis* **25**(6), 685-8.

References

Basmaciogullari, S., Babcock, G. J., Van Ryk, D., Wojtowicz, W., and Sodroski, J. (2002). Identification of conserved and variable structures in the human immunodeficiency virus gp120 glycoprotein of importance for CXCR4 binding. *J Virol* **76**(21), 10791-800.

Belshe, R. B., Clements, M. L., Dolin, R., Graham, B. S., McElrath, J., Gorse, G. J., Schwartz, D., Keefer, M. C., Wright, P., Corey, L., and et al. (1993). Safety and immunogenicity of a fully glycosylated recombinant gp160 human immunodeficiency virus type 1 vaccine in subjects at low risk of infection. National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group Network. *J Infect Dis* **168**(6), 1387-95.

Belshe, R. B., Gorse, G. J., Mulligan, M. J., Evans, T. G., Keefer, M. C., Excler, J. L., Duliege, A. M., Tartaglia, J., Cox, W. I., McNamara, J., Hwang, K. L., Bradney, A., Montefiori, D., and Weinhold, K. J. (1998). Induction of immune responses to HIV-1 by canarypox virus (ALVAC) HIV-1 and gp120 SF-2 recombinant vaccines in uninfected volunteers. NIAID AIDS Vaccine Evaluation Group. *Aids* **12**(18), 2407-15.

Benichou, S., Legrand, R., Nakagawa, N., Faure, T., Traincard, F., Vogt, G., Dormont, D., Tiollais, P., Kieny, M. P., and Madaule, P. (1992). Identification of a neutralizing domain in the external envelope glycoprotein of simian immunodeficiency virus. *AIDS Res Hum Retroviruses* **8**(6), 1165-70.

Benjamin, J., Ganser-Pornillos, B. K., Tivol, W. F., Sundquist, W. I., and Jensen, G. J. (2004). Three-dimensional Structure of HIV-1 Virus-like Particles by Electron Cryotomography. *Journal of Molecular Biology* **346**, 577-588.

Benn, S., Rutledge, R., Folks, T., Gold, J., Baker, L., McCormick, J., Feorino, P., Piot, P., Quinn, T., and Martin, M. (1985). Genomic heterogeneity of AIDS retroviral isolates from North America and Zaire. *Science* **230**(4728), 949-51.

Bentz, J., and Mittal, A. (2003). Architecture of the influenza hemagglutinin membrane fusion site. *Biochim Biophys Acta* **1614**(1), 24-35.

Berger, E. A., Doms, R. W., Fenyo, E. M., Korber, B. T., Littman, D. R., Moore, J. P., Sattentau, Q. J., Schuitemaker, H., Sodroski, J., and Weiss, R. A. (1998). A new classification for HIV-1. *Nature* **391**(6664), 240.

Berger, E. A., Lifson, J. D., and Eiden, L. E. (1991). Stimulation of glycoprotein gp120 dissociation from the envelope glycoprotein complex of human immunodeficiency virus type 1 by soluble CD4 and CD4 peptide derivatives: implications for the role of the complementarity-determining region 3-like region in membrane fusion. *Proc Natl Acad Sci U S A* **88**(18), 8082-6.

Berger, E. A., Murphy, P. M., and Farber, J. M. (1999). Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* **17**, 657-700.

Bernstein, H. B., and Compans, R. W. (1992). Sulfation of the Human Immunodeficiency Virus Envelope Glycoprotein. *Journal of Virology* **66**(12), 6953-6959.

Berry, N., Ariyoshi, K., Jaffar, S., Sabally, S., Corrah, T., Tedder, R., and Whittle, H. (1998). Low peripheral blood viral HIV-2 RNA in individuals with high CD4 percentage differentiates HIV-2 from HIV-1 infection. *J Hum Virol* **1**(7), 457-68.

Bertoletti, A., Cham, F., McAdam, S., Rostron, T., Rowland-Jones, S., Sabally, S., Corrah, T., Ariyoshi, K., and Whittle, H. (1998). Cytotoxic T cells from human immunodeficiency virus type 2-infected patients frequently cross-react with different human immunodeficiency virus type 1 clades. *J Virol* **72**(3), 2439-48.

Bhattacharya, J., Peters, P. J., and Clapham, P. R. (2003). CD4-independent infection of HIV and SIV: implications for envelope conformation and cell tropism in vivo. *AIDS* **17 Suppl 4**, S35-43.

Bibollet-Ruche, F., Bailes, E., Gao, F., Pourrut, X., Barlow, K. L., Clewley, J. P., Mwenda, J. M., Langat, D. K., Chege, G. K., McClure, H. M., Mpoudi-Ngole, E., Delaporte, E., Peeters, M., Shaw, G. M., Sharp, P. M., and Hahn, B. H. (2004). New simian immunodeficiency virus infecting De Brazza's monkeys (Cercopithecus neglectus): evidence for a cercopithecus monkey virus clade. *J Virol* **78**(14), 7748-62.

Billich, A. (2003). S-1360 Shionogi-GlaxoSmithKline. *Curr Opin Investig Drugs* **4**(2), 206-9.

Binley, J. M., Sanders, R. W., Master, A., Cayanan, C. S., Wiley, C. L., Schiffner, L., Travis, B., Kuhmann, S., Burton, D. R., Hu, S. L., Olson, W. C., and Moore, J. P. (2002). Enhancing the proteolytic maturation of human immunodeficiency virus type 1 envelope glycoproteins. *J Virol* **76**(6), 2606-16.

References

Bjorndal, A., Deng, H., Jansson, M., Fiore, J. R., Colognesi, C., Karlsson, A., Albert, J., Scarlatti, G., Littman, D. R., and Fenyo, E. M. (1997). Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J Virol* **71**(10), 7478-87.

Blaak, H., Boers, P. H., Schutten, M., van der Enden, M. E., and Osterhaus, A. D. (2004). HIV-2-infected individuals with undetectable plasma viremia carry replication-competent virus in peripheral blood lymphocytes. *J Acquir Immune Defic Syndr* **36**(3), 777-82.

Blackbourn, D. J., Locher, C. P., Ramachandran, B., Barnett, S. W., Murthy, K. K., Carey, K. D., Brasky, K. M., and Levy, J. A. (1997). CD8+ cells from HIV-2-infected baboons control HIV replication. *AIDS* **11**(6), 737-46.

Blacklow, S. C., Lu, M., and Kim, P. S. (1995). A trimeric subdomain of the simian immunodeficiency virus envelope glycoprotein. *Biochemistry* **34**(46), 14955-62.

Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T. A. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* **382**(6594), 829-33.

Bock, P. J., and Markovitz, D. M. (2001). Infection with HIV-2. *AIDS* **15**(5), S35-S45.

Bodelle, P., Vallari, A., Coffey, R., McArthur, C. P., Beyeme, M., Devare, S. G., Schochetman, G., and Brennan, C. A. (2004). Identification and genomic sequence of an HIV type 1 group N isolate from Cameroon. *AIDS Res Hum Retroviruses* **20**(8), 902-8.

Boden, D., Hurley, A., Zhang, L., Cao, Y., Guo, Y., Jones, E., Tsay, J., Ip, J., Farthing, C., Limoli, K., Parkin, N., and Markowitz, M. (1999). HIV-1 drug resistance in newly infected individuals. *Jama* **282**(12), 1135-41.

Boeri, E., Giri, A., Lillo, F., Ferrari, G., Varnier, O. E., Ferro, A., Sabbatani, S., Saxinger, W. C., and Franchini, G. (1992). In vivo genetic variability of the human immunodeficiency virus type 2 V3 region. *J Virol* **66**(7), 4546-50.

Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M., and Oldstone, M. B. (1994). Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* **68**(9), 6103-10.

Borrow, P., Lewicki, H., Wei, X., Horwitz, M. S., Peffer, N., Meyers, H., Nelson, J. A., Gairin, J. E., Hahn, B. H., Oldstone, M. B., and Shaw, G. M. (1997). Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat Med* **3**(2), 205-11.

Botarelli, P., Houlden, B. A., Haigwood, N. L., Servis, C., Montagna, D., and Abrignani, S. (1991). N-glycosylation of HIV-gp120 may constrain recognition by T lymphocytes. *J Immunol* **147**(9), 3128-32.

Bour, S., Akari, H., Miyagi, E., and Strebel, K. (2003). Naturally occurring amino acid substitutions in the HIV-2 ROD envelope glycoprotein regulate its ability to augment viral particle release. *Virology* **309**(1), 85-98.

Bour, S., Geleziunas, R., and Wainberg, M. A. (1995). The human immunodeficiency virus type 1 (HIV-1) CD4 receptor and its central role in promotion of HIV-1 infection. *Microbiol Rev* **59**(1), 63-93.

Bour, S., Schubert, U., Peden, K., and Strebel, K. (1996). The envelope glycoprotein of human immunodeficiency virus type 2 enhances viral particle release: a Vpu-like factor? *J Virol* **70**(2), 820-9.

Bour, S., Schubert, U., and Strebel, K. (1995). The human immunodeficiency virus type 1 Vpu protein specifically binds to the cytoplasmic domain of CD4: implications for the mechanism of degradation. *J Virol* **69**(3), 1510-20.

Bour, S., and Strebel, K. (1996). The human immunodeficiency virus (HIV) type 2 envelope protein is a functional complement to HIV type 1 Vpu that enhances particle release of heterologous retroviruses. *J Virol* **70**(12), 8285-300.

Bour, S. P., Aberham, C., Perrin, C., and Strebel, K. (1999). Lack of effect of cytoplasmic tail truncations on human immunodeficiency virus type 2 ROD env particle release activity. *J Virol* **73**(1), 778-82.

Bower, J. F., Li, Y., Wyatt, R., and Ross, T. M. (2006). HIV-1 Envgp140 trimers elicit neutralizing antibodies without efficient induction of conformational antibodies. *Vaccine* **24**(26), 5442-51.

Bower, J. F., Yang, X., Sodroski, J., and Ross, T. M. (2004). Elicitation of Neutralising Antibodies with DNA Vaccines Expressing Soluble Stabilised Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Trimers Conjugated to C3d. *Journal of Virology* **78**(9), 4710-4719.

References

Brasseur, R. (1991). Differentiation of lipid-associating helices by use of three-dimensional molecular hydrophobicity potential calculations. *J Biol Chem* **266**(24), 16120-7.

Breen, E. C., Rezai, A. R., Nakajima, K., Beall, G. N., Mitsuyasu, R. T., Hirano, T., Kishimoto, T., and Martinez-Maza, O. (1990). Infection with HIV is associated with elevated IL-6 levels and production. *J Immunol* **144**(2), 480-4.

Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., Nguyen, P. L., Khoruts, A., Larson, M., Haase, A. T., and Douek, D. C. (2004). CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* **200**(6), 749-59.

Brenner, B. G., Routy, J. P., Petrella, M., Moisi, D., Oliveira, M., Detorio, M., Spira, B., Essabag, V., Conway, B., Lalonde, R., Sekaly, R. P., and Wainberg, M. A. (2002). Persistence and fitness of multidrug-resistant human immunodeficiency virus type 1 acquired in primary infection. *J Virol* **76**(4), 1753-61.

Briggs, J. A., Johnson, M. C., Simon, M. N., Fuller, S. D., and Vogt, V. M. (2006). Cryo-electron microscopy reveals conserved and divergent features of gag packing in immature particles of Rous sarcoma virus and human immunodeficiency virus. *J Mol Biol* **355**(1), 157-68.

Broder, C. C., Dimitrov, D. S., Blumenthal, R., and Berger, E. A. (1993). The block to HIV-1 envelope glycoprotein-mediated membrane fusion in animal cells expressing human CD4 can be overcome by a human cell component(s). *Virology* **193**(1), 483-91.

Brodsky, M. H., Warton, M., Myers, R. M., and Littman, D. R. (1990). Analysis of the site in CD4 that binds to the HIV envelope glycoprotein. *J Immunol* **144**(8), 3078-86.

Bron, R., Klasse, P. J., Wilkinson, D., Clapham, P. R., Pelchen-Matthews, A., Power, C., Wells, T. N. C., Kim, J., Peiper, S. C., Hoxie, J. A., and Marsh, M. (1997). Promiscuous Use of CC and CXC Chemokine Receptors in Cell-to-Cell Fusion Mediated by a Human Immunodeficiency Virus Type 2 Envelope Protein. *Journal of Virology* **71**(11), 8405-8415.

Brown, P. O. (1990). Integration of retroviral DNA. *Curr Top Microbiol Immunol* **157**, 19-48.

Buchacher, A., Predl, R., Strutzenberger, K., Steinfellner, W., Trkola, A., Purtscher, M., Gruber, G., Tauer, C., Steindl, F., Jungbauer, A., and et al. (1994). Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. *AIDS Res Hum Retroviruses* **10**(4), 359-69.

Buckheit, R. W., Jr., Watson, K., Fliakas-Boltz, V., Russell, J., Loftus, T. L., Osterling, M. C., Turpin, J. A., Pallansch, L. A., White, E. L., Lee, J. W., Lee, S. H., Oh, J. W., Kwon, H. S., Chung, S. G., and Cho, E. H. (2001). SJ-3366, a unique and highly potent nonnucleoside reverse transcriptase inhibitor of human immunodeficiency virus type 1 (HIV-1) that also inhibits HIV-2. *Antimicrob Agents Chemother* **45**(2), 393-400.

Burton, D. R., Desrosiers, R. C., Doms, R. W., Koff, W. C., Kwong, P. D., Moore, J. P., Nabel, G. J., Sodroski, J., Wilson, I. A., and Wyatt, R. T. (2004). HIV vaccine design and the neutralizing antibody problem. *Nat Immunol* **5**(3), 233-6.

Burton, D. R., Pyati, J., Koduri, R., Sharp, S. J., Thornton, G. B., Parren, P. W., Sawyer, L. S., Hendry, R. M., Dunlop, N., Nara, P. L., and et al. (1994). Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. *Science* **266**(5187), 1024-7.

Caffrey, M., Cai, M., Kaufman, J., Stahl, S. J., Wingfield, P. T., Covell, D. G., Gronenborn, A. M., and Clore, G. M. (1998). Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41. *Embo J* **17**(16), 4572-84.

Calarese, D. A., Lee, H. K., Huang, C. Y., Best, M. D., Astronomo, R. D., Stanfield, R. L., Katinger, H., Burton, D. R., Wong, C. H., and Wilson, I. A. (2005). Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. *Proc Natl Acad Sci U S A* **102**(38), 13372-7.

Calarese, D. A., Scanlan, C. N., Zwick, M. B., Deechongkit, S., Mimura, Y., Kunert, R., Zhu, P., Wormald, M. R., Stanfield, R. L., Roux, K. H., Kelly, J. W., Rudd, P. M., Dwek, R. A., Katinger, H., Burton, D. R., and Wilson, I. A. (2003). Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. *Science* **300**(5628), 2065-71.

Camaur, D., and Trono, D. (1996). Characterization of human immunodeficiency virus type 1 Vif particle incorporation. *J Virol* **70**(9), 6106-11.

Campbell, S. M., Crowe, S. M., and Mak, J. (2001). Lipid rafts and HIV-1: from viral entry to assembly of progeny virions. *J Clin Virol* **22**(3), 217-27.

Cao, J., Bergeron, L., Helseth, E., Thali, M., Repke, H., and Sodroski, J. (1993). Effects of amino acid changes in the extracellular domain of the human immunodeficiency virus type 1 gp41 envelope glycoprotein. *J Virol* **67**(5), 2747-55.

Cao, J., Park, I. W., Cooper, A., and Sodroski, J. (1996). Molecular determinants of acute single-cell lysis by human immunodeficiency virus type 1. *J Virol* **70**(3), 1340-54.

Cao, Y., Qin, L., Zhang, L., Safrit, J., and Ho, D. D. (1995). Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N Engl J Med* **332**(4), 201-8.

Carr, A., and Cooper, D. A. (2000). Adverse effects of antiretroviral therapy. *Lancet* **356**(9239), 1423-30.

Carr, A., Samaras, K., Thorisdottir, A., Kaufmann, G. R., Chisholm, D. J., and Cooper, D. A. (1999). Diagnosis, prediction, and natural course of HIV-1 protease-inhibitor-associated lipodystrophy, hyperlipidaemia, and diabetes mellitus: a cohort study. *Lancet* **353**(9170), 2093-9.

Carrington, M., Nelson, G. W., Martin, M. P., Kissner, T., Vlahov, D., Goedert, J. J., Kaslow, R., Buchbinder, S., Hoots, K., and O'Brien, S. J. (1999). HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science* **283**(5408), 1748-52.

Castano, S., and Desbat, B. (2005). Structure and orientation study of fusion peptide FP23 of gp41 from HIV-1 alone or inserted into various lipid membrane models (mono-, bi- and multibi-layers) by FT-IR spectroscopies and Brewster angle microscopy. *Biochim Biophys Acta* **1715**(2), 81-95.

Castro, K. G., Ward, J. W., Slutsker, L., Buehler, J. W., Jaffe, H. W., Berkelman, R. L., and Curran, J. W. (1992). Centres for Disease Control and Prevention. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *Morbidity and Mortality Weekly Report* **41**, 1-19.

Cavaco-Silva, P., Taveira, N. C., Lourenco, M. H., Santos Ferreira, M. O., and Daniels, R. S. (1997). Vertical transmission of HIV-2. *Lancet* **349**(9046), 177-8.

Cavaco-Silva, P., Taveira, N. C., Rosado, L., Lourenco, M. H., Moniz-Pereira, J., Douglas, N. W., Daniels, R. S., and Santos-Ferreira, M. O. (1998). Virological and molecular demonstration of human immunodeficiency virus type 2 vertical transmission. *J Virol* **72**(4), 3418-22.

Cavaleiro, R., Sousa, A. E., Loureiro, A., and Victorino, R. M. (2000). Marked immunosuppressive effects of the HIV-2 envelope protein in spite of the lower HIV-2 pathogenicity. *Aids* **14**(17), 2679-86.

Cazein, F., Hamers, F., Alix, J., and Brunet, J. B. (1996). Prevalence of HIV-2 infection in Europe. *Euro Surveill* **1**(3), 21-23.

Cecilia, D., Kleeberger, C., Munoz, A., Giorgi, J. V., and Zolla-Pazner, S. (1999). A longitudinal study of neutralizing antibodies and disease progression in HIV-1-infected subjects. *J Infect Dis* **179**(6), 1365-74.

Center, R. J., Lebowitz, J., Leapman, R. D., and Moss, B. (2004). Promoting trimerization of soluble human immunodeficiency virus type 1 (HIV-1) Env through the use of HIV-1/simian immunodeficiency virus chimeras. *J Virol* **78**(5), 2265-76.

Center, R. J., Schuck, P., Leapman, R. D., Arthur, L. O., Earl, P. L., Moss, B., and Lebowitz, J. (2001). Oligomeric structure of virion-associated and soluble forms of the simian immunodeficiency virus envelope protein in the prefusion activated conformation. *Proc Natl Acad Sci U S A* **98**(26), 14877-82.

Chakrabarti, B. K., Kong, W., Wu, B., Yang, Z., Friborg, J., Ling, X., King, S. R., Montefiori, D. C., and Nabel, G. J. (2002). Modifications of the Human Immunodeficiency Virus Envelope Glycoprotein Enhance Immunogenicity for Genetic Immunization. *Journal of Virology* **76**(11), 5357-5368.

Chakrabarti, L., Guyader, M., Alizon, M., Daniel, M. D., Desrosiers, R. C., Tiollais, P., and Sonigo, P. (1987). Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. *Nature* **328**(6130), 543-7.

Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997). Core Structure of gp41 from the HIV Envelope Glycoprotein. *Cell* **89**, 263-273.

Chan, D. C., and Kim, P. S. (1998). HIV entry and its inhibition. *Cell* **93**(5), 681-4.

Chapman, B. S., Thayer, R. M., Vincent, K. A., and Haigwood, N. L. (1991). Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Res* **19**(14), 3979-86.

References

Chastel, C., and Charmot, G. (2004). [Bacterial and viral epidemics of zoonotic origin; the role of hunting and cutting up wild animals]. *Bull Soc Pathol Exot* **97**(3), 207-12.

Chen, B., Vogan, E. M., Gong, H., Skehel, J. J., Wiley, D. C., and Harrison, S. C. (2005a). Determining the Structure of an Unliganded and Fully Glycosylated SIV gp120 Envelope Glycoprotein. *Structure (Camb)* **13**(2), 197-211.

Chen, B., Vogan, E. M., Gong, H., Skehel, J. J., Wiley, D. C., and Harrison, S. C. (2005b). Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature* **433**(7028), 834-41.

Chen, B., Zhou, G., Kim, M., Chishti, Y., Hussey, R. E., Ely, B., Skehel, J. J., Reinherz, E. L., Harrison, S. C., and Wiley, D. C. (2000). Expression, purification, and characterization of gp160e, the soluble, trimeric ectodomain of the simian immunodeficiency virus envelope glycoprotein, gp160. *J Biol Chem* **275**(45), 34946-53.

Chen, Z., Kwon, D., Jin, Z., Monard, S., Telfer, P., Jones, M. S., Lu, C. Y., Aguilar, R. F., Ho, D. D., and Marx, P. A. (1998). Natural infection of a homozygous delta24 CCR5 red-capped mangabey with an R2b-tropic simian immunodeficiency virus. *J Exp Med* **188**(11), 2057-65.

Chen, Z., Luckay, A., Sodora, D. L., Telfer, P., Reed, P., Gettie, A., Kanu, J. M., Sadek, R. F., Yee, J., Ho, D. D., Zhang, L., and Marx, P. A. (1997). Human immunodeficiency virus type 2 (HIV-2) seroprevalence and characterization of a distinct HIV-2 genetic subtype from the natural range of simian immunodeficiency virus-infected sooty mangabeys. *J Virol* **71**(5), 3953-60.

Chernomordik, L. V., Melikyan, G. B., and Chizmadzhev, Y. A. (1987). Biomembrane fusion: a new concept derived from model studies using two interacting planar lipid bilayers. *Biochim Biophys Acta* **906**(3), 309-52.

Chertova, E., Bess Jr, J. W., Jr., Crise, B. J., Sowder, I. R., Schaden, T. M., Hilburn, J. M., Hoxie, J. A., Benveniste, R. E., Lifson, J. D., Henderson, L. E., and Arthur, L. O. (2002). Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), Is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus. *J Virol* **76**(11), 5315-25.

Chesebro, B., Buller, R., Portis, J., and Wehrly, K. (1990). Failure of human immunodeficiency virus entry and infection in CD4-positive human brain and skin cells. *J Virol* **64**(1), 215-21.

Chirmule, N., Kalyanaraman, V. S., Oyaizu, N., Slade, H. B., and Pahwa, S. (1990). Inhibition of functional properties of tetanus antigen-specific T-cell clones by envelope glycoprotein GP120 of human immunodeficiency virus. *Blood* **75**(1), 152-9.

Chirmule, N., and Pahwa, S. (1996). Envelope Glycoproteins of Human Immunodeficiency Virus Type 1: Profound Influences on Immune Functions. *Microbiological Reviews* **60**(2), 386-406.

Chiu, T. K., and Davies, D. R. (2004). Structure and function of HIV-1 integrase. *Curr Top Med Chem* **4**(9), 965-77.

Cho, M. W., Lee, M. K., Carney, M. C., Berson, J. F., Doms, R. W., and Martin, M. A. (1998). Identification of determinants on a dualtropic human immunodeficiency virus type 1 envelope glycoprotein that confer usage of CXCR4. *J Virol* **72**(3), 2509-15.

Choe, H., Farzan, M., Konkel, M., Martin, K., Sun, Y., Marcon, L., Cayabyab, M., Berman, M., Dorf, M. E., Gerard, N., Gerard, C., and Sodroski, J. (1998). The orphan seven-transmembrane receptor apj supports the entry of primary T-cell-line-tropic and dualtropic human immunodeficiency virus type 1. *J Virol* **72**(7), 6113-8.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and Sodroski, J. (1996). The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**(7), 1135-48.

Choe, H. R., and Sodroski, J. (1992). Contribution of charged amino acids in the CDR2 region of CD4 to HIV-1 gp120 binding. *J Acquir Immune Defic Syndr* **5**(2), 204-10.

Cicala, C., Arthos, J., Ruiz, M., Vaccarezza, M., Rubbert, A., Riva, A., Wildt, K., Cohen, O., and Fauci, A. S. (1999). Induction of phosphorylation and intracellular association of CC chemokine receptor 5 and focal adhesion kinase in primary human CD4+ T cells by macrophage-tropic HIV envelope. *J Immunol* **163**(1), 420-6.

Cilla, G., Rodes, B., Perez-Trallero, E., Arrizabalaga, J., and Soriano, V. (2001). Molecular evidence of homosexual transmission of HIV type 2 in Spain. *AIDS Res Hum Retroviruses* **17**(5), 417-22.

Clapham, P. R., Blanc, D., and Weiss, R. A. (1991). Specific cell surface requirements for the infection of CD4-positive cells by human immunodeficiency virus types 1 and 2 and by Simian immunodeficiency virus. *Virology* **181**(2), 703-15.

Clapham, P. R., McKnight, A., and Weiss, R. A. (1992). Human immunodeficiency virus type 2 infection and fusion of CD4-negative human cell lines: induction and enhancement by soluble CD4. *J Virol* **66**(6), 3531-7.

Clark, S. J., Jefferies, W. A., Barclay, A. N., Gagnon, J., and Williams, A. F. (1987). Peptide and nucleotide sequences of rat CD4 (W3/25) antigen: evidence for derivation from a structure with four immunoglobulin-related domains. *Proc Natl Acad Sci U S A* **84**(6), 1649-53.

Clavel, F., Brun-Vezinet, F., Guetard, D., Chamaret, S., Laurent, A., Rouzioux, C., Rey, M., Katlama, C., Rey, F., Champelinaud, J. L., and et al. (1986a). [LAV type II: a second retrovirus associated with AIDS in West Africa]. *C R Acad Sci III* **302**(13), 485-8.

Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M. A., Santos-Ferreira, M. O., Laurent, A. G., Dauguet, C., Katlama, C., Rouzioux, C., and et al. (1986b). Isolation of a new human retrovirus from West African patients with AIDS. *Science* **233**(4761), 343-6.

Clavel, F., Guyader, M., Guetard, D., Salle, M., Montagnier, L., and Alizon, M. (1986c). Molecular cloning and polymorphism of the human immune deficiency virus type 2. *Nature* **324**(6098), 691-5.

Clayton, L. K., Sieh, M., Pious, D. A., and Reinherz, E. L. (1989). Identification of human CD4 residues affecting class II MHC versus HIV-1 gp120 binding. *Nature* **339**(6225), 548-51.

Clements, G. J., Price-Jones, M. J., Stephens, P. E., Sutton, C., Schulz, T. F., Clapham, P. R., McKeating, J. A., McClure, M. O., Thomson, S., Marsh, M., and et al. (1991). The V3 loops of the HIV-1 and HIV-2 surface glycoproteins contain proteolytic cleavage sites: a possible function in viral fusion? *AIDS Res Hum Retroviruses* **7**(1), 3-16.

Coates, J. A., Cammack, N., Jenkinson, H. J., Jowett, A. J., Jowett, M. I., Pearson, B. A., Penn, C. R., Rouse, P. L., Viner, K. C., and Cameron, J. M. (1992). (-)-2'-deoxy-3'-thiacytidine is a potent, highly selective inhibitor of human immunodeficiency virus type 1 and type 2 replication in vitro. *Antimicrob Agents Chemother* **36**(4), 733-9.

Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995). Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270**(5243), 1811-5.

Cohen, E. A., Subbramanian, R. A., and Gottlinger, H. G. (1996). Role of auxiliary proteins in retroviral morphogenesis. *Curr Top Microbiol Immunol* **214**, 219-35.

Cohen, O. J., Kinter, A., and Fauci, A. S. (1997). Host factors in the pathogenesis of HIV disease. *Immunol Rev* **159**, 31-48.

Cohen Stuart, J. W., Hazebergh, M. D., Hamann, D., Otto, S. A., Borleffs, J. C., Miedema, F., Boucher, C. A., and de Boer, R. J. (2000). The dominant source of CD4+ and CD8+ T-cell activation in HIV infection is antigenic stimulation. *J Acquir Immune Defic Syndr* **25**(3), 203-11.

Cole, J. L., and Hansen, J. C. (1999). Analytical Ultracentrifugation as a contemporary Biomolecular Research Tool. *Journal of Biomolecular Techniques* **10**(4), 163-176.

Colebunders, R., De Vuyst, H., Verstraeten, T., Schroyens, W., and Van Marck, E. (1995). A non-Hodgkin's lymphoma in a patient with HIV-2 infection. *Genitourin Med* **71**(2), 129.

Collier, A. C., Coombs, R. W., Schoenfeld, D. A., Bassett, R. L., Timpone, J., Baruch, A., Jones, M., Facey, K., Whitacre, C., McAuliffe, V. J., Friedman, H. M., Merigan, T. C., Reichman, R. C., Hooper, C., and Corey, L. (1996). Treatment of human immunodeficiency virus infection with saquinavir, zidovudine, and zalcitabine. AIDS Clinical Trials Group. *N Engl J Med* **334**(16), 1011-7.

Colman, P. M., and Lawrence, M. C. (2003). The structural biology of type I viral membrane fusion. *Nat Rev Mol Cell Biol* **4**(4), 309-19.

Connor, R. I., and Ho, D. D. (1994). Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. *J Virol* **68**(7), 4400-8.

Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S., and Landau, N. R. (1997). Change in coreceptor use coreceptor use correlates with disease progression in HIV-1--infected individuals. *J Exp Med* **185**(4), 621-8.

Cortes, E., Detels, R., Aboulafia, D., Li, X. L., Moudgil, T., Alam, M., Bonecker, C., Gonzaga, A., Oyafuso, L., and Tondo, M. (1989). HIV-1, HIV-2, and HTLV-I infection in high-risk groups in Brazil. *N Engl J Med* **320**(15), 953-8.

Cox, S. W., Aperia, K., Albert, J., and Wahren, B. (1994). Comparison of the sensitivities of primary isolates of HIV type 2 and HIV type 1 to antiviral drugs and drug combinations. *AIDS Res Hum Retroviruses* **10**(12), 1725-9.

Craven, R. C., and Parent, L. J. (1996). Dynamic interactions of the Gag polyprotein. *Curr Top Microbiol Immunol* **214**, 65-94.

Critchlow, C. W., and Kiviat, N. B. (1997). Detection of human immunodeficiency virus type 1 and type 2 in the female genital tract: implications for the understanding of virus transmission. *Obstet Gynecol Surv* **52**(5), 315-24.

Daar, E. S., Li, X. L., Moudgil, T., and Ho, D. D. (1990). High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. *Proc Natl Acad Sci U S A* **87**(17), 6574-8.

Dalgleish, A. G., Beverley, P. C., Clapham, P. R., Crawford, D. H., Greaves, M. F., and Weiss, R. A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**(5996), 763-7.

Damond, F., Apetrei, C., Robertson, D. L., Souquiere, S., Lepretre, A., Matheron, S., Plantier, J. C., Brun-Vezinet, F., and Simon, F. (2001). Variability of human immunodeficiency virus type 2 (hiv-2) infecting patients living in france. *Virology* **280**(1), 19-30.

Damond, F., Worobey, M., Campa, P., Farfara, I., Colin, G., Matheron, S., Brun-Vezinet, F., Robertson, D. L., and Simon, F. (2004). Identification of a highly divergent HIV type 2 and proposal for a change in HIV type 2 classification. *AIDS Res Hum Retroviruses* **20**(6), 666-72.

Daniel, M. D., Letvin, N. L., King, N. W., Kannagi, M., Sehgal, P. K., Hunt, R. D., Kanki, P. J., Essex, M., and Desrosiers, R. C. (1985). Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* **228**(4704), 1201-4.

D'Arcy, A. (1994). Crystallizing proteins - a rational approach? *Acta Crystallogr D Biol Crystallogr* **50**(Pt 4), 469-71.

Datema, R., Rabin, L., Hincenbergs, M., Moreno, M. B., Warren, S., Linquist, V., Rosenwirth, B., Seifert, J., and McCune, J. M. (1996). Antiviral efficacy in vivo of the anti-human immunodeficiency virus bicyclam SDZ SID 791 (JM 3100), an inhibitor of infectious cell entry. *Antimicrob Agents Chemother* **40**(3), 750-4.

Davis, S. J., Ward, H. A., Puklavec, M. J., Willis, A. C., Williams, A. F., and Barclay, A. N. (1990). High level expression in Chinese hamster ovary cells of soluble forms of CD4 T lymphocyte glycoprotein including glycosylation variants. *J Biol Chem* **265**(18), 10410-8.

Dayton, A. I., Sodroski, J. G., Rosen, C. A., Goh, W. C., and Haseltine, W. A. (1986). The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. *Cell* **44**(6), 941-7.

De Clercq, E., Yamamoto, N., Pauwels, R., Balzarini, J., Witvrouw, M., De Vreese, K., Debyser, Z., Rosenwirth, B., Peichl, P., Datema, R., and et al. (1994). Highly potent and selective inhibition of human immunodeficiency virus by the bicyclam derivative JM3100. *Antimicrob Agents Chemother* **38**(4), 668-74.

De Cock, K. M., Adjorlolo, G., Ekpini, E., Sibailly, T., Kouadio, J., Maran, M., Brattegaard, K., Vetter, K. M., Doorly, R., and Gayle, H. D. (1993). Epidemiology and transmission of HIV-2. Why there is no HIV-2 pandemic. *Jama* **270**(17), 2083-6.

De Cock, K. M., Gnaore, E., Adjorlolo, G., Braun, M. M., Lafontaine, M. F., Yesso, G., Bretton, G., Coulibaly, I. M., Gershy-Damet, G. M., Bretton, R., and et al. (1991). Risk of tuberculosis in patients with HIV-I and HIV-II infections in Abidjan, Ivory Coast. *Bmj* **302**(6775), 496-9.

Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Smith, M. W., Allikmets, R., Goedert, J. J., Buchbinder, S. P., Vittinghoff, E., Gomperts, E., Donfield, S., Vlahov, D., Kaslow, R., Saah, A., Rinaldo, C., Detels, R., and O'Brien, S. J. (1996). Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* **273**(5283), 1856-62.

Decroly, E., Cornet, B., Martin, I., Ruysschaert, J. M., and Vandenbranden, M. (1993). Secondary structure of gp160 and gp120 envelope glycoproteins of human immunodeficiency virus type 1: a Fourier transform infrared spectroscopic study. *J Virol* **67**(6), 3552-60.

Delahunty, M. D., Rhee, I., Freed, E. O., and Bonifacino, J. S. (1996). Mutational analysis of the fusion peptide of the human immunodeficiency virus type 1: identification of critical glycine residues. *Virology* **218**(1), 94-102.

Delaporte, E., Janssens, W., Peeters, M., Buve, A., Dibanga, G., Perret, J. L., Ditsambou, V., Mba, J. R., Courbot, M. C., Georges, A., Bourgeois, A., Samb, B., Henzel, D., Heyndrickx, L., Fransen, K., van der Groen, G., and Larouze, B. (1996). Epidemiological and molecular characteristics of HIV infection in Gabon, 1986-1994. *AIDS* **10**(8), 903-10.

Demarchi, F., d'Adda di Fagagna, F., Falaschi, A., and Giacca, M. (1996). Activation of transcription factor NF-kappaB by the Tat protein of human immunodeficiency virus type 1. *J Virol* **70**(7), 4427-37.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R., and Landau, N. R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**(6584), 661-6.

Deng, H. K., Unutmaz, D., KewalRamani, V. N., and Littman, D. R. (1997). Expression cloning of new receptors used by simian and human immunodeficiency viruses. *Nature* **388**(6639), 296-300.

Deora, A., Spearman, P., and Ratner, L. (2000). The N-terminal matrix domain of HIV-1 Gag is sufficient but not necessary for viral protein U-mediated enhancement of particle release through a membrane-targeting mechanism. *Virology* **269**(2), 305-12.

Derdeyn, C. A., and Silvestri, G. (2005). Viral and host factors in the pathogenesis of HIV infection. *Curr Opin Immunol* **17**(4), 366-73.

Dimitrov, A. S., Xiao, X., Dimitrov, D. S., and Blumenthal, R. (2001). Early intermediates in HIV-1 envelope glycoprotein-mediated fusion triggered by CD4 and co-receptor complexes. *J Biol Chem* **276**(32), 30335-41.

Dimitrov, D. S., Golding, H., and Blumenthal, R. (1991). Initial stages of HIV-1 envelope glycoprotein-mediated cell fusion monitored by a new assay based on redistribution of fluorescent dyes. *AIDS Res Hum Retroviruses* **7**(10), 799-805.

Dimitrov, D. S., Hillman, K., Manischewitz, J., Blumenthal, R., and Golding, H. (1992). Correlation between kinetics of soluble CD4 interactions with HIV-1-Env-expressing cells and inhibition of syncytia formation: implications for mechanisms of cell fusion and therapy for AIDS. *AIDS* **6**(3), 249-56.

Dimitrov, D. S., Willey, R. L., Sato, H., Chang, L. J., Blumenthal, R., and Martin, M. A. (1993). Quantitation of human immunodeficiency virus type 1 infection kinetics. *J Virol* **67**(4), 2182-90.

Doms, R. W. (2000). Beyond receptor expression: the influence of receptor conformation, density, and affinity in HIV-1 infection. *Virology* **276**(2), 229-37.

Doranz, B. J., Filion, L. G., Diaz-Mitoma, F., Sitar, D. S., Sahai, J., Baribaud, F., Orsini, M. J., Benovic, J. L., Cameron, W., and Doms, R. W. (2001). Safe use of the CXCR4 inhibitor ALX40-4C in humans. *AIDS Res Hum Retroviruses* **17**(6), 475-86.

Doranz, B. J., Grovit-Ferbas, K., Sharron, M. P., Mao, S. H., Goetz, M. B., Daar, E. S., Doms, R. W., and O'Brien, W. A. (1997). A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. *J Exp Med* **186**(8), 1395-400.

Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G., and Doms, R. W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**(7), 1149-58.

Dorrell, L., Willcox, B. E., Jones, E. Y., Gillespie, G., Njai, H., Sabally, S., Jaye, A., DeGleria, K., Rostron, T., Lepin, E., McMichael, A., Whittle, H., and Rowland-Jones, S. (2001). Cytotoxic T lymphocytes recognize structurally diverse, clade-specific and cross-reactive peptides in human immunodeficiency virus type-1 gag through HLA-B53. *Eur J Immunol* **31**(6), 1747-56.

Dougan, S., Patel, B., Tosswill, J. H., and Sinka, K. (2005). Diagnoses of HIV-1 and HIV-2 in England, Wales, and Northern Ireland associated with west Africa. *Sex Transm Infect* **81**(4), 338-41.

Douglas, N. W., Knight, A. I., Hayhurst, A., Barrett, W. Y., Kevany, M. J., and Daniels, R. S. (1996). An efficient method for the rescue and analysis of functional HIV-1 env genes: evidence for recombination in the vicinity of the tat/rev splice site. *AIDS* **10**(1), 39-46.

References

Douglas, N. W., Munro, G. H., and Daniels, R. S. (1997). HIV/SIV Glycoproteins: Structure-Function
Relationships. *Journal of Molecular Biology* **273**, 122-149.

Dragic, T., Charneau, P., Clavel, F., and Alizon, M. (1992). Complementation of murine cells for human immunodeficiency virus envelope/CD4-mediated fusion in human/murine heterokaryons. *J Virol* **66**(8), 4794-802.

Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., and Paxton, W. A. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**(6584), 667-73.

Dragic, T., Trkola, A., Thompson, D. A., Cormier, E. G., Kajumo, F. A., Maxwell, E., Lin, S. W., Ying, W., Smith, S. O., Sakmar, T. P., and Moore, J. P. (2000). A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc Natl Acad Sci U S A* **97**(10), 5639-44.

Dufoort, G., Courouce, A. M., Ancelle-Park, R., and Bletry, O. (1988). No clinical signs 14 years after HIV-2 transmission via blood transfusion. *Lancet* **2**(8609), 510.

Dumonceaux, J., Nisole, S., Chanel, C., Quivet, L., Amara, A., Baleux, F., Briand, P., and Hazan, U. (1998). Spontaneous mutations in the env gene of the human immunodeficiency virus type 1 NDK isolate are associated with a CD4-independent entry phenotype. *J Virol* **72**(1), 512-9.

Earl, P. L., Sugiura, W., Montefiori, D. C., Broder, C. C., Lee, S. A., Wild, C., Lifson, J., and Moss, B. (2001). Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140. *J Virol* **75**(2), 645-53.

Edinger, A. L., Hoffman, T. L., Sharron, M., Lee, B., Yi, Y., Choe, W., Kolson, D. L., Mitrovic, B., Zhou, Y., Faulds, D., Collman, R. G., Hesselgesser, J., Horuk, R., and Doms, R. W. (1998). An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. *J Virol* **72**(10), 7934-40.

Edwards, T. G., Wyss, S., Reeves, J. D., Zolla-Pazner, S., Hoxie, J. A., Doms, R. W., and Baribaud, F. (2002). Truncation of the cytoplasmic domain induces exposure of conserved regions in the ectodomain of human immunodeficiency virus type 1 envelope protein. *J Virol* **76**(6), 2683-91.

Egan, M. A., Charini, W. A., Kuroda, M. J., Schmitz, J. E., Racz, P., Tenner-Racz, K., Manson, K., Wyand, M., Lifton, M. A., Nickerson, C. E., Fu, T., Shiver, J. W., and Letvin, N. L. (2000). Simian immunodeficiency virus (SIV) gag DNA-vaccinated rhesus monkeys develop secondary cytotoxic T-lymphocyte responses and control viral replication after pathogenic SIV infection. *J Virol* **74**(16), 7485-95.

Elbein, A. D. (1991). Glycosidase inhibitors: inhibitors of N-linked oligosaccharide processing. *Faseb J* **5**(15), 3055-63.

Ellerbrok, H., D'Auriol, L., Vaquero, C., and Sitbon, M. (1992). Functional tolerance of the human immunodeficiency virus type 1 envelope signal peptide to mutations in the amino-terminal and hydrophobic regions. *J Virol* **66**(8), 5114-8.

Embrey, M. W., Wai, J. S., Funk, T. W., Homnick, C. F., Perlow, D. S., Young, S. D., Vacca, J. P., Hazuda, D. J., Felock, P. J., Stillmock, K. A., Witmer, M. V., Moyer, G., Schleif, W. A., Gabryelski, L. J., Jin, L., Chen, I. W., Ellis, J. D., Wong, B. K., Lin, J. H., Leonard, Y. M., Tsou, N. N., and Zhuang, L. (2005). A series of 5-(5,6)-dihydrouracil substituted 8-hydroxy-[1,6]naphthyridine-7-carboxylic acid 4-fluorobenzylamide inhibitors of HIV-1 integrase and viral replication in cells. *Bioorg Med Chem Lett* **15**(20), 4550-4.

Emerman, M. (1996). HIV-1, Vpr and the cell cycle. *Curr Biol* **6**(9), 1096-103.

Emerman, M., Vazeux, R., and Peden, K. (1989). The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. *Cell* **57**(7), 1155-65.

Engelman, A., Mizuuchi, K., and Craigie, R. (1991). HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. *Cell* **67**(6), 1211-21.

Epstein, L. G., Kuiken, C., Blumberg, B. M., Hartman, S., Sharer, L. R., Clement, M., and Goudsmit, J. (1991). HIV-1 V3 domain variation in brain and spleen of children with AIDS: tissue-specific evolution within host-determined quasispecies. *Virology* **180**(2), 583-90.

Excler, J. L., and Plotkin, S. (1997). The prime-boost concept applied to HIV preventive vaccines. *AIDS* **11 Suppl A**, S127-37.

References

Fanales-Belasio, E., Cafaro, A., Cara, A., Negri, D. R., Fiorelli, V., Butto, S., Moretti, S., Maggiorella, M. T., Baroncelli, S., Michelini, Z., Tripiciano, A., Sernicola, L., Scoglio, A., Borsetti, A., Ridolfi, B., Bona, R., Ten Haaft, P., Macchia, I., Leone, P., Pavone-Cossut, M. R., Nappi, F., Vardas, E., Magnani, M., Laguardia, E., Caputo, A., Titti, F., and Ensoli, B. (2002). HIV-1 Tat-based vaccines: from basic science to clinical trials. *DNA Cell Biol* **21**(9), 599-610.

Farzan, M., Choe, H., Martin, K., Marcon, L., Hofmann, W., Karlsson, G., Sun, Y., Barrett, P., Marchand, N., Sullivan, N., Gerard, N., Gerard, C., and Sodroski, J. (1997). Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection. *J Exp Med* **186**(3), 405-11.

Fauci, A. S. (1988). The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. *Science* **239**(4840), 617-22.

Feinberg, M. B., Jarrett, R. F., Aldovini, A., Gallo, R. C., and Wong-Staal, F. (1986). HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. *Cell* **46**(6), 807-17.

Felser, J. M., Klimkait, T., and Silver, J. (1989). A syncytia assay for human immunodeficiency virus type I (HIV-I) envelope protein and its use in studying HIV-I mutations. *Virology* **170**(2), 566-70.

Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**(5263), 872-7.

Fenouillet, E., Miquelis, R., and Drillien, R. (1996). Biological properties of recombinant HIV envelope synthesized in CHO glycosylation-mutant cell lines. *Virology* **218**(1), 224-31.

Ferrantelli, F., Rasmussen, R. A., Buckley, K. A., Li, P. L., Wang, T., Montefiori, D. C., Katinger, H., Stiegler, G., Anderson, D. C., McClure, H. M., and Ruprecht, R. M. (2004). Complete protection of neonatal rhesus macaques against oral exposure to pathogenic simian-human immunodeficiency virus by human anti-HIV monoclonal antibodies. *J Infect Dis* **189**(12), 2167-73.

Ferre-D'Amare, A., and Burley, S. (1997). Dynamic Light Scattering in Evaluating Crystallizability of Macromolecules. *Methods in Enzymology* **276**, 157-166.

Finnegan, C. M., Berg, W., Lewis, G. K., and DeVico, A. L. (2001). Antigenic properties of the human immunodeficiency virus envelope during cell-cell fusion. *J Virol* **75**(22), 11096-105.

Fisher, A. G., Feinberg, M. B., Josephs, S. F., Harper, M. E., Marselle, L. M., Reyes, G., Gonda, M. A., Aldovini, A., Debouk, C., Gallo, R. C., and et al. (1986). The trans-activator gene of HTLV-III is essential for virus replication. *Nature* **320**(6060), 367-71.

Fletcher, T. M., 3rd, Brichacek, B., Sharova, N., Newman, M. A., Stivahtis, G., Sharp, P. M., Emerman, M., Hahn, B. H., and Stevenson, M. (1996). Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV(SM). *Embo J* **15**(22), 6155-65.

Fleury, S., Lamarre, D., Meloche, S., Ryu, S. E., Cantin, C., Hendrickson, W. A., and Sekaly, R. P. (1991). Mutational analysis of the interaction between CD4 and class II MHC: class II antigens contact CD4 on a surface opposite the gp120-binding site. *Cell* **66**(5), 1037-49.

Flint, J., Sims, M., Clark, K., Staden, R., and Thomas, K. (1998). An oligo-screening strategy to fill gaps found during shotgun sequencing projects. *DNA Seq* **8**(4), 241-5.

Fowke, K. R., D'Amico, R., Chernoff, D. N., Pottage, J. C., Jr., Benson, C. A., Sha, B. E., Kessler, H. A., Landay, A. L., and Shearer, G. M. (1997). Immunologic and virologic evaluation after influenza vaccination of HIV-1-infected patients. *AIDS* **11**(8), 1013-21.

Franchini, G., Gurgo, C., Guo, H. G., Gallo, R. C., Collalti, E., Fargnoli, K. A., Hall, L. F., Wong-Staal, F., and Reitz, M. S., Jr. (1987). Sequence of simian immunodeficiency virus and its relationship to the human immunodeficiency viruses. *Nature* **328**(6130), 539-43.

Francis, D. P., Curran, J. W., and Essex, M. (1983). Epidemic acquired immune deficiency syndrome: epidemiologic evidence for a transmissible agent. *J Natl Cancer Inst* **71**(1), 1-4.

Freed, E. O., Myers, D. J., and Risser, R. (1990). Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. *Proc Natl Acad Sci U S A* **87**(12), 4650-4.

Frey, S., Marsh, M., Gunther, S., Pelchen-Matthews, A., Stephens, P., Ortlepp, S., and Stegmann, T. (1995). Temperature dependence of cell-cell fusion induced by the envelope glycoprotein of human immunodeficiency virus type 1. *J Virol* **69**(3), 1462-72.

Friedman-Kien, A., Laubenstein, L., Marmor, M., Hymes, K., Green, J., Ragaz, A., Gottlieb, J., Muggia, F., Demopoulos, R., Weintraub, M., Williams, D., Oliveri, R., Marmer, J., Wallace, J., Halperin, I., Gillooley, J. F., Prose, N., Klein, E., Vogel, J., Safai, B., Myskowski, P., Urmacher, C., Koziner, B., Nisce, L., Kris, M., Armstrong, D., Gold, J., Mildran, D., Tapper, M., Weissman, J. B., Rothenberg, R., Friedman, S. M., F.P., S., Groundwater, J., Gilmore, J., Coleman, D., Follansbee, S., Gullet, J., Stegman, S. J., Wofsy, C., Bush, D., Drew, L., Braff, E., Dritz, S., Klein, M., Preiksaitis, J. K., Gottlieb, M. S., Jung, R., Chin, J., and Goedert, J. J. (1981). Kaposi's Sarcoma and *Pneumocystis* Pneumonia among homosexual men - New York City and California. *Morbidity and Mortality Weekly Report* **30**(25), 305-308.

Furuta, R. A., Wild, C. T., Weng, Y., and Weiss, C. D. (1998). Capture of an early fusion-active conformation of HIV-1 gp41. *Nat Struct Biol* **5**(4), 276-9.

Fust, G. (1997). Enhancing antibodies in HIV infection. *Parasitology* **115 Suppl**, S127-40.

Gahery, H., Choppin, J., Bourgault, I., Fischer, E., Maillere, B., and Guillet, J. G. (2005). HIV preventive vaccine research at the ANRS: the lipopeptide vaccine approach. *Therapie* **60**(3), 243-8.

Gahery-Segard, H., Pialoux, G., Figueiredo, S., Igea, C., Surenaud, M., Gaston, J., Gras-Masse, H., Levy, J. P., and Guillet, J. G. (2003). Long-term specific immune responses induced in humans by a human immunodeficiency virus type 1 lipopeptide vaccine: characterization of CD8+-T-cell epitopes recognized. *J Virol* **77**(20), 11220-31.

Gallo, R. C., and Montagnier, L. (1987). The chronology of AIDS research. *Nature* **326**(6112), 435-6.

Gallo, R. C., Sarin, P. S., Gelmann, E. P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V. S., Mann, D., Sidhu, G. D., Stahl, R. E., Zolla-Pazner, S., Leibowitch, J., and Popovic, M. (1983). Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* **220**(4599), 865-7.

Gallo, S. A., Puri, A., and Blumenthal, R. (2001). HIV-1 gp41 six-helix bundle formation occurs rapidly after the engagement of gp120 by CXCR4 in the HIV-1 Env-mediated fusion process. *Biochemistry* **40**(41), 12231-6.

Gao, F., Bailes, E., Robertson, D. L., Chen, Y., Rodenburg, C. M., Michael, S. F., Cummins, L. B., Arthur, L. O., Peeters, M., Shaw, G. M., Sharp, P. M., and Hahn, B. H. (1999). Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397**(6718), 436-41.

Gao, F., Robertson, D. L., Carruthers, C. D., Morrison, S. G., Jian, B., Chen, Y., Barre-Sinoussi, F., Girard, M., Srinivasan, A., Abimiku, A. G., Shaw, G. M., Sharp, P. M., and Hahn, B. H. (1998). A comprehensive panel of near-full-length clones and reference sequences for non-subtype B isolates of human immunodeficiency virus type 1. *J Virol* **72**(7), 5680-98.

Gao, F., Yue, L., Robertson, D. L., Hill, S. C., Hui, H., Biggar, R. J., Neequaye, A. E., Whelan, T. M., Ho, D. D., Shaw, G. M., and et al. (1994). Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. *J Virol* **68**(11), 7433-47.

Gao, F., Yue, L., White, A. T., Pappas, P. G., Barchue, J., Hanson, A. P., Greene, B. M., Sharp, P. M., Shaw, G. M., and Hahn, B. H. (1992). Human infection by genetically diverse SIVSM-related HIV-2 in west Africa. *Nature* **358**(6386), 495-9.

Garlick, R. L., Kirschner, R. J., Eckenrode, F. M., Tarpley, W. G., and Tomich, C. C. (1990). Escherichia coli Expression, Purification, and Biological Activity of a Truncated Soluble CD4. *AIDS Research and Human Rertroviruses* **6**(4), 465-479.

Gaschen, B., Taylor, J., Yusim, K., Foley, B., Gao, F., Lang, D., Novitsky, V., Haynes, B., Hahn, B. H., Bhattacharya, T., and Korber, B. (2002). Diversity considerations in HIV-1 vaccine selection. *Science* **296**(5577), 2354-60.

Gauduin, M. C., Allaway, G. P., Maddon, P. J., Barbas, C. F., 3rd, Burton, D. R., and Koup, R. A. (1996). Effective ex vivo neutralization of human immunodeficiency virus type 1 in plasma by recombinant immunoglobulin molecules. *J Virol* **70**(4), 2586-92.

Gauduin, M. C., Allaway, G. P., Olson, W. C., Weir, R., Maddon, P. J., and Koup, R. A. (1998). CD4-immunoglobulin G2 protects Hu-PBL-SCID mice against challenge by primary human immunodeficiency virus type 1 isolates. *J Virol* **72**(4), 3475-8.

Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G., and van Kooyk, Y. (2000). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**(5), 587-97.

Gelderblom, H. R., Hausmann, E. H., Ozel, M., Pauli, G., and Koch, M. A. (1987). Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. *Virology* **156**(1), 171-6.

Gelderblom, H. R., Ozel, M., and Pauli, G. (1989). Morphogenesis and morphology of HIV. Structure-function relations. *Arch Virol* **106**(1-2), 1-13.

Gelderblom, H. R., Reupke, H., and Pauli, G. (1985). Loss of envelope antigens of HTLV-III/LAV, a factor in AIDS pathogenesis? *Lancet* **2**(8462), 1016-7.

Gershoni, J. M., Denisova, G., Raviv, D., Smorodinsky, N. I., and Buyaner, D. (1993). HIV binding to its receptor creates specific epitopes for the CD4/gp120 complex. *Faseb J* **7**(12), 1185-7.

Ghys, P. D., Fransen, K., Diallo, M. O., Ettiegne-Traore, V., Coulibaly, I. M., Yeboue, K. M., Kalish, M. L., Maurice, C., Whitaker, J. P., Greenberg, A. E., and Laga, M. (1997). The associations between cervicovaginal HIV shedding, sexually transmitted diseases and immunosuppression in female sex workers in Abidjan, Cote d'Ivoire. *Aids* **11**(12), F85-93.

Gilbert, P. B., Ackers, M. L., Berman, P. W., Francis, D. P., Popovic, V., Hu, D. J., Heyward, W. L., Sinangil, F., Shepherd, B. E., and Gurwith, M. (2005a). HIV-1 virologic and immunologic progression and initiation of antiretroviral therapy among HIV-1-infected subjects in a trial of the efficacy of recombinant glycoprotein 120 vaccine. *J Infect Dis* **192**(6), 974-83.

Gilbert, P. B., Peterson, M. L., Follmann, D., Hudgens, M. G., Francis, D. P., Gurwith, M., Heyward, W. L., Jobes, D. V., Popovic, V., Self, S. G., Sinangil, F., Burke, D., and Berman, P. W. (2005b). Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. *J Infect Dis* **191**(5), 666-77.

Gilljam, G. (1993). Envelope glycoproteins of HIV-1, HIV-2, and SIV purified with Galanthus nivalis agglutinin induce strong immune responses. *AIDS Res Hum Retroviruses* **9**(5), 431-8.

Giorgi, J. V., Hausner, M. A., and Hultin, L. E. (1999). Detailed immunophenotype of CD8+ memory cytotoxic T-lymphocytes (CTL) against HIV-1 with respect to expression of CD45RA/RO, CD62L and CD28 antigens. *Immunol Lett* **66**(1-3), 105-10.

Giorgi, J. V., Lyles, R. H., Matud, J. L., Yamashita, T. E., Mellors, J. W., Hultin, L. E., Jamieson, B. D., Margolick, J. B., Rinaldo, C. R., Jr., Phair, J. P., and Detels, R. (2002). Predictive value of immunologic and virologic markers after long or short duration of HIV-1 infection. *J Acquir Immune Defic Syndr* **29**(4), 346-55.

Giri, M., Ugen, K. E., and Weiner, D. B. (2004). DNA vaccines against human immunodeficiency virus type 1 in the past decade. *Clin Microbiol Rev* **17**(2), 370-89.

Gloster, S. E., Newton, P., Cornforth, D., Lifson, J. D., Williams, I., Shaw, G. M., and Borrow, P. (2004). Association of strong virus-specific CD4 T cell responses with efficient natural control of primary HIV-1 infection. *AIDS* **18**(5), 749-55.

Godbole, S., and Mehendale, S. (2005). HIV/AIDS epidemic in India: risk factors, risk behaviour & strategies for prevention & control. *Indian J Med Res* **121**(4), 356-68.

Goff, S. P. (1992). Genetics of retroviral integration. *Annu Rev Genet* **26**, 527-44.

Golden, A., Austen, D. A., van Schravendijk, M. R., Sullivan, B. J., Kawasaki, E. S., and Osburne, M. S. (1998). Effect of promoters and signal sequences on the production of secreted HIV-1 gp120 protein in the baculovirus system. *Protein Expr Purif* **14**(1), 8-12.

Gonda, M. A., Wong-Staal, F., Gallo, R. C., Clements, J. E., Narayan, O., and Gilden, R. V. (1985). Sequence homology and morphologic similarity of HTLV-III and visna virus, a pathogenic lentivirus. *Science* **227**(4683), 173-7.

Gorman, C. M., Howard, B. H., and Reeves, R. (1983). Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res* **11**(21), 7631-48.

Gorny, M. K., Conley, A. J., Karwowska, S., Buchbinder, A., Xu, J. Y., Emini, E. A., Koenig, S., and Zolla-Pazner, S. (1992). Neutralization of diverse human immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. *J Virol* **66**(12), 7538-42.

Gorny, M. K., Williams, C., Volsky, B., Revesz, K., Wang, X. H., Burda, S., Kimura, T., Konings, F. A., Nadas, A., Anyangwe, C. A., Nyambi, P., Krachmarov, C., Pinter, A., and Zolla-Pazner, S. (2006). Cross-clade neutralizing activity of human anti-V3 monoclonal antibodies derived from the cells of individuals infected with non-B clades of human immunodeficiency virus type 1. *J Virol* **80**(14), 6865-72.

Gorry, P. R., Churchill, M., Crowe, S. M., Cunningham, A. L., and Gabuzda, D. (2005). Pathogenesis of macrophage tropic HIV-1. *Curr HIV Res* **3**(1), 53-60.

Gorry, P. R., Taylor, J., Holm, G. H., Mehle, A., Morgan, T., Cayabyab, M., Farzan, M., Wang, H., Bell, J. E., Kunstman, K., Moore, J. P., Wolinsky, S. M., and Gabuzda, D. (2002). Increased CCR5 affinity and reduced CCR5/CD4 dependence of a neurovirulent primary human immunodeficiency virus type 1 isolate. *J Virol* **76**(12), 6277-92.

Gottlieb, M. S., Schanker, H. M., Fan, P. T., Saxon, A., Weisman, J. D., and Pozalski, I. (1981a). *Pneumocystis* Pneumonia - Los Angeles. *Morbidity and Mortality Weekly Report* **30**(21), 250-252.

Gottlieb, M. S., Schroff, R., Schanker, H. M., Weisman, J. D., Fan, P. T., Wolf, R. A., and Saxon, A. (1981b). Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med* **305**(24), 1425-31.

Gottlinger, H. G., Dorfman, T., Cohen, E. A., and Haseltine, W. A. (1993). Vpu protein of human immunodeficiency virus type 1 enhances the release of capsids produced by gag gene constructs of widely divergent retroviruses. *Proc Natl Acad Sci U S A* **90**(15), 7381-5.

Gougeon, M. L., Lecoeur, H., Dulioust, A., Enouf, M. G., Crouvoiser, M., Goujard, C., Debord, T., and Montagnier, L. (1996). Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression. *J Immunol* **156**(9), 3509-20.

Goulder, P. J., Phillips, R. E., Colbert, R. A., McAdam, S., Ogg, G., Nowak, M. A., Giangrande, P., Luzzi, G., Morgan, B., Edwards, A., McMichael, A. J., and Rowland-Jones, S. (1997). Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* **3**(2), 212-7.

Gram, G. J., Hemming, A., Bolmstedt, A., Jansson, B., Olofsson, S., Akerblom, L., Nielsen, J. O., and Hansen, J. E. (1994). Identification of an N-linked glycan in the V1-loop of HIV-1 gp120 influencing neutralization by anti-V3 antibodies and soluble CD4. *Arch Virol* **139**(3-4), 253-61.

Grant, A. D., Djomand, G., and De Cock, K. M. (1997). Natural history and spectrum of disease in adults with HIV/AIDS in Africa. *AIDS* **11 Suppl B**, S43-54.

Grant, A. D., Djomand, G., Smets, P., Kadio, A., Coulibaly, M., Kakou, A., Maurice, C., Whitaker, J. P., Sylla-Koko, F., Bonard, D., Wiktor, S. Z., Hayes, R. J., De Cock, K. M., and Greenberg, A. E. (1997). Profound immunosuppression across the spectrum of opportunistic disease among hospitalized HIV-infected adults in Abidjan, Cote d'Ivoire. *AIDS* **11**(11), 1357-64.

Grassly, N. C., Xiang, Z., Ariyoshi, K., Aaby, P., Jensen, H., Schim van der Loeff, M., Dias, F., Whittle, H., and Breuer, J. (1998). Mortality among human immunodeficiency virus type 2-positive villagers in rural Guinea-Bissau is correlated with viral genotype. *J Virol* **72**(10), 7895-9.

Greenberg, A. E. (2001). Possible protective effect of HIV-2 against incident HIV-1 infection: review of available epidemiological and in vitro data. *AIDS* **15**(17), 2319-21.

Greenblatt, R. M., Lukehart, S. A., Plummer, F. A., Quinn, T. C., Critchlow, C. W., Ashley, R. L., D'Costa, L. J., Ndinya-Achola, J. O., Corey, L., Ronald, A. R., and et al. (1988). Genital ulceration as a risk factor for human immunodeficiency virus infection. *AIDS* **2**(1), 47-50.

Grossman, Z., Meier-Schellersheim, M., Sousa, A. E., Victorino, R. M., and Paul, W. E. (2002). CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause? *Nat Med* **8**(4), 319-23.

Grossman, Z., Polis, M., Feinberg, M. B., Levi, I., Jankelevich, S., Yarchoan, R., Boon, J., de Wolf, F., Lange, J. M., Goudsmit, J., Dimitrov, D. S., and Paul, W. E. (1999). Ongoing HIV dissemination during HAART. *Nat Med* **5**(10), 1099-104.

Grundner, C., Li, Y., Louder, M., Mascola, J., Yang, X., Sodroski, J., and Wyatt, R. (2005). Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins. *Virology* **331**(1), 33-46.

Guadalupe, M., Reay, E., Sankaran, S., Prindiville, T., Flamm, J., McNeil, A., and Dandekar, S. (2003). Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* **77**(21), 11708-17.

Guillon, C., van der Ende, M. E., Boers, P. H., Gruters, R. A., Schutten, M., and Osterhaus, A. D. (1998). Coreceptor usage of human immunodeficiency virus type 2 primary isolates and biological clones is broad and does not correlate with their syncytium-inducing capacities. *J Virol* **72**(7), 6260-3.

Gulick, R. M., Mellors, J. W., Havlir, D., Eron, J. J., Gonzalez, C., McMahon, D., Richman, D. D., Valentine, F. T., Jonas, L., Meibohm, A., Emini, E. A., and Chodakewitz, J. A. (1997). Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. *N Engl J Med* **337**(11), 734-9.

Gurtler, L. G., Hauser, P. H., Eberle, J., von Brunn, A., Knapp, S., Zekeng, L., Tsague, J. M., and Kaptue, L. (1994). A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. *J Virol* **68**(3), 1581-5.

Guyader, M., Emerman, M., Sonigo, P., Clavel, F., Montagnier, L., and Alizon, M. (1987). Genome organization and transactivation of the human immunodeficiency virus type 2. *Nature* **326**(6114), 662-9.

Haase, A. T. (2005). Perils at mucosal front lines for HIV and SIV and their hosts. *Nat Rev Immunol* **5**(10), 783-92.

Haggerty, S., Dempsey, M. P., Bukrinsky, M. I., Guo, L., and Stevenson, M. (1991). Posttranslational modifications within the HIV-1 envelope glycoprotein which restrict virus assembly and CD4-dependent infection. *AIDS Res Hum Retroviruses* **7**(6), 501-10.

Hallenberger, S., Moulard, M., Sordel, M., Klenk, H. D., and Garten, W. (1997). The role of eukaryotic subtilisin-like endoproteases for the activation of human immunodeficiency virus glycoproteins in natural host cells. *J Virol* **71**(2), 1036-45.

Hammer, S. M., Squires, K. E., Hughes, M. D., Grimes, J. M., Demeter, L. M., Currier, J. S., Eron, J. J., Jr., Feinberg, J. E., Balfour, H. H., Jr., Deyton, L. R., Chodakewitz, J. A., and Fischl, M. A. (1997). A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N Engl J Med* **337**(11), 725-33.

Hanke, T., Samuel, R. V., Blanchard, T. J., Neumann, V. C., Allen, T. M., Boyson, J. E., Sharpe, S. A., Cook, N., Smith, G. L., Watkins, D. I., Cranage, M. P., and McMichael, A. J. (1999). Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. *J Virol* **73**(9), 7524-32.

Hannibal, M. C., Markovitz, D. M., Clark, N., and Nabel, G. J. (1993). Differential activation of human immunodeficiency virus type 1 and 2 transcription by specific T-cell activation signals. *J Virol* **67**(8), 5035-40.

Hansen, J. E., Lund, O., Nielsen, J. O., and Brunak, S. (1996). Prediction of the secondary structure of HIV-1 gp120. *Proteins* **25**(1), 1-11.

Hanson, A. D. S., A. Shea, A. Jones, N. Mboup, S. Kanki, P. Cao, H (2005). Distinct Profile of T Cell Activation in HIV Type 2 Compared to HIV Type 1 Infection: Differential Mechanism for Immunoprotection. *AIDS Research and HUman Rertroviruses* **21**(9), 791-798.

Harris, R. S., Bishop, K. N., Sheehy, A. M., Craig, H. M., Petersen-Mahrt, S. K., Watt, I. N., Neuberger, M. S., and Malim, M. H. (2003). DNA deamination mediates innate immunity to retroviral infection. *Cell* **113**(6), 803-9.

Harter, C., James, P., Bachi, T., Semenza, G., and Brunner, J. (1989). Hydrophobic binding of the ectodomain of influenza hemagglutinin to membranes occurs through the "fusion peptide". *J Biol Chem* **264**(11), 6459-64.

Hawkes, S., West, B., Wilson, S., Whittle, H., and Mabey, D. (1995). Asymptomatic carriage of Haemophilus ducreyi confirmed by the polymerase chain reaction. *Genitourin Med* **71**(4), 224-7.

Hazenberg, M. D., Hamann, D., Schuitemaker, H., and Miedema, F. (2000). T cell depletion in HIV-1 infection: how CD4+ T cells go out of stock. *Nat Immunol* **1**(4), 285-9.

Hazenberg, M. D., Otto, S. A., van Benthem, B. H., Roos, M. T., Coutinho, R. A., Lange, J. M., Hamann, D., Prins, M., and Miedema, F. (2003). Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS* **17**(13), 1881-8.

Hazuda, D. J., Felock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J. A., Espeseth, A., Gabryelski, L., Schleif, W., Blau, C., and Miller, M. D. (2000). Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science* **287**(5453), 646-50.

He, Y., Vassell, R., Zaitseva, M., Nguyen, N., Yang, Z., Weng, Y., and Weiss, C. D. (2003). Peptides trap the human immunodeficiency virus type 1 envelope glycoprotein fusion intermediate at two sites. *J Virol* **77**(3), 1666-71.

Helseth, E., Olshevsky, U., Furman, C., and Sodroski, J. (1991). Human immunodeficiency virus type 1 gp120 envelope glycoprotein regions important for association with the gp41 transmembrane glycoprotein. *J Virol* **65**(4), 2119-23.

Hendel, H., Caillat-Zucman, S., Lebuanec, H., Carrington, M., O'Brien, S., Andrieu, J. M., Schachter, F., Zagury, D., Rappaport, J., Winkler, C., Nelson, G. W., and Zagury, J. F. (1999). New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS. *J Immunol* **162**(11), 6942-6.

Henderson, L. E., Sowder, R. C., Copeland, T. D., Benveniste, R. E., and Oroszlan, S. (1988). Isolation and characterization of a novel protein (X-ORF product) from SIV and HIV-2. *Science* **241**(4862), 199-201.

Hendrix, C. W., Collier, A. C., Lederman, M. M., Schols, D., Pollard, R. B., Brown, S., Jackson, J. B., Coombs, R. W., Glesby, M. J., Flexner, C. W., Bridger, G. J., Badel, K., MacFarland, R. T., Henson, G. W., and Calandra, G. (2004). Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. *J Acquir Immune Defic Syndr* **37**(2), 1253-62.

Herrera, A. M., Musacchio, A., Fernandez, J. R., and Duarte, C. A. (2000). Efficiency of erythropoietin's signal peptide for HIV(MN)-1 gp 120 expression. *Biochem Biophys Res Commun* **273**(2), 557-9.

Hinkula, J., Gidlund, M., Persson, C., Osterhaus, A., and Wahren, B. (1994). Enzyme immunoassay (ELISA) for the evaluation of antibodies directed to the CD4 receptor-binding site of the HIV gp120 molecule. *J Immunol Methods* **175**(1), 37-46.

Hira, S. K., Kamanga, J., Bhat, G. J., Mwale, C., Tembo, G., Luo, N., and Perine, P. L. (1989). Perinatal transmission of HIV-I in Zambia. *Bmj* **299**(6710), 1250-2.

Hira, S. K., Mangrola, U. G., Mwale, C., Chintu, C., Tembo, G., Brady, W. E., and Perine, P. L. (1990). Apparent vertical transmission of human immunodeficiency virus type 1 by breast-feeding in Zambia. *J Pediatr* **117**(3), 421-4.

Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H., and Johnson, P. R. (1989). An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339**(6223), 389-92.

Hizi, A., Tal, R., Shaharabany, M., Currens, M. J., Boyd, M. R., Hughes, S. H., and McMahon, J. B. (1993). Specific inhibition of the reverse transcriptase of human immunodeficiency virus type 1 and the chimeric enzymes of human immunodeficiency virus type 1 and type 2 by nonnucleoside inhibitors. *Antimicrob Agents Chemother* **37**(5), 1037-42.

Hoffman, T. L., and Doms, R. W. (1999). HIV-1 envelope determinants for cell tropism and chemokine receptor use. *Mol Membr Biol* **16**(1), 57-65.

Hoffman, T. L., LaBranche, C. C., Zhang, W., Canziani, G., Robinson, J., Chaiken, I., Hoxie, J. A., and Doms, R. W. (1999). Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein. *Proc Natl Acad Sci U S A* **96**(11), 6359-64.

Hoffman, T. L., Stephens, E. B., Narayan, O., and Doms, R. W. (1998). HIV type I envelope determinants for use of the CCR2b, CCR3, STRL33, and APJ coreceptors. *Proc Natl Acad Sci U S A* **95**(19), 11360-5.

Hogan, C. M., and Hammer, S. M. (2001). Host determinants in HIV infection and disease. Part 2: genetic factors and implications for antiretroviral therapeutics. *Ann Intern Med* **134**(10), 978-96.

Holmberg, S. D., Horsburgh, C. R., Jr., Ward, J. W., and Jaffe, H. W. (1989). Biologic factors in the sexual transmission of human immunodeficiency virus. *J Infect Dis* **160**(1), 116-25.

Hosmalin, A., Andrieu, M., Loing, E., Desoutter, J. F., Hanau, D., Gras-Masse, H., Dautry-Varsat, A., and Guillet, J. G. (2001). Lipopeptide presentation pathway in dendritic cells. *Immunol Lett* **79**(1-2), 97-100.

Hotzel, I., and Cheevers, W. (2002). Differential receptor usage of small ruminant lentiviruses in ovine and caprine cells: host range but not cytopathic phenotype is determined by receptor usage. *Virology* **301**(1), 21-31.

Houlgatte, R., Scarmato, P., el Marhomy, S., Martin, M., Ostankovitch, M., Lafosse, S., Vervisch, A., Auffray, C., and Platier-Tonneau, D. (1994). HLA class II antigens and the HIV envelope glycoprotein gp120 bind to the same face of CD4. *J Immunol* **152**(9), 4475-88.

Hoxie, J. A. (1991). Hypothetical assignment of Intrachain Disulphide Bonds for HIV-2 and SIV Envelope Glycoproteins. *AIDS Research and Human Retroviruses* **7**(6), 495-499.

Hu, Q., Trent, J. O., Tomaras, G. D., Wang, Z., Murray, J. L., Conolly, S. M., Navenot, J. M., Barry, A. P., Greenberg, M. L., and Peiper, S. C. (2000). Identification of ENV determinants in V3 that influence the molecular anatomy of CCR5 utilization. *J Mol Biol* **302**(2), 359-75.

Huang, C. C., Tang, M., Zhang, M. Y., Majeed, S., Montabana, E., Stanfield, R. L., Dimitrov, D. S., Korber, B., Sodroski, J., Wilson, I. A., Wyatt, R., and Kwong, P. D. (2005). Structure of a V3-containing HIV-1 gp120 core. *Science* **310**(5750), 1025-8.

Huang, Q., Sivaramakrishna, R. P., Ludwig, K., Korte, T., Bottcher, C., and Herrmann, A. (2003). Early steps of the conformational change of influenza virus hemagglutinin to a fusion active state: stability and energetics of the hemagglutinin. *Biochim Biophys Acta* **1614**(1), 3-13.

Huang, Y., Paxton, W. A., Wolinsky, S. M., Neumann, A. U., Zhang, L., He, T., Kang, S., Ceradini, D., Jin, Z., Yazdanbakhsh, K., Kunstman, K., Erickson, D., Dragon, E., Landau, N. R., Phair, J., Ho, D. D., and Koup, R. A. (1996). The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat Med* **2**(11), 1240-3.

Huet, T., Cheynier, R., Meyerhans, A., Roelants, G., and Wain-Hobson, S. (1990). Genetic organization of a chimpanzee lentivirus related to HIV-1. *Nature* **345**(6273), 356-9.

Hunt, J. C., Brennan, C. A., Golden, A. M., Yamaguchi, J., Lund, J. K., Vallari, A. S., Hickman, R. K., Zekeng, L., Gurtler, L. G., Hampl, H., Kaptue, L., and Devare, S. G. (1997). Molecular analyses of HIV-1 group O and HIV-2 variants from Africa. *Leukemia* **11 Suppl 3**, 138-41.

Hurwitz, J. L., Slobod, K. S., Lockey, T. D., Wang, S., Chou, T. H., and Lu, S. (2005). Application of the polyvalent approach to HIV-1 vaccine development. *Curr Drug Targets Infect Disord* **5**(2), 143-56.

Igarashi, T., Brown, C., Azadegan, A., Haigwood, N., Dimitrov, D., Martin, M. A., and Shibata, R. (1999). Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma. *Nat Med* **5**(2), 211-6.

Isaka, Y., Sato, A., Miki, S., Kawauchi, S., Sakaida, H., Hori, T., Uchiyama, T., Adachi, A., Hayami, M., Fujiwara, T., and Yoshie, O. (1999). Small amino acid changes in the V3 loop of human immunodeficiency virus type 2 determines the coreceptor usage for CXCR4 and CCR5. *Virology* **264**(1), 237-43.

Isel, C., Ehresmann, C., Walter, P., Ehresmann, B., and Marquet, R. (2001). The emergence of different resistance mechanisms toward nucleoside inhibitors is explained by the properties of the wild type HIV-1 reverse transcriptase. *J Biol Chem* **276**(52), 48725-32.

Ishikawa, K., Janssens, W., Banor, J. S., Shinno, T., Piedade, J., Sata, T., Ampofo, W. K., Brandful, J. A., Koyanagi, Y., Yamamoto, N., Canas-Ferreira, W. F., Adu-Sarkodie, Y., and Kurata, T. (2001). Genetic analysis of HIV type 2 from Ghana and Guinea-Bissau, West Africa. *AIDS Res Hum Retroviruses* **17**(17), 1661-3.

Itescu, S., Simonelli, P. F., Winchester, R. J., and Ginsberg, H. S. (1994). Human immunodeficiency virus type 1 strains in the lungs of infected individuals evolve independently from those in peripheral blood and are highly conserved in the C-terminal region of the envelope V3 loop. *Proc Natl Acad Sci U S A* **91**(24), 11378-82.

Ivey-Hoyle, M., Culp, J. S., Chaikin, M. A., Hellmig, B. D., Matthews, T. J., Sweet, R. W., and Rosenberg, M. (1991). Envelope glycoproteins from biologically diverse isolates of immunodeficiency viruses have widely different affinities for CD4. *Proc Natl Acad Sci U S A* **88**(2), 512-6.

Jacobson, J. M., Lowy, I., Fletcher, C. V., O'Neill, T. J., Tran, D. N., Ketas, T. J., Trkola, A., Klotman, M. E., Maddon, P. J., Olson, W. C., and Israel, R. J. (2000). Single-dose safety, pharmacology, and antiviral activity of the human immunodeficiency virus (HIV) type 1 entry inhibitor PRO 542 in HIV-infected adults. *J Infect Dis* **182**(1), 326-9.

Jaffar, S., Wilkins, A., Ngom, P. T., Sabally, S., Corrah, T., Bangali, J. E., Rolfe, M., and Whittle, H. C. (1997). Rate of decline of percentage CD4+ cells is faster in HIV-1 than in HIV-2 infection. *J Acquir Immune Defic Syndr Hum Retrovirol* **16**(5), 327-32.

Jameson, B. A., Rao, P. E., Kong, L. I., Hahn, B. H., Shaw, G. M., Hood, L. E., and Kent, S. B. (1988). Location and chemical synthesis of a binding site for HIV-1 on the CD4 protein. *Science* **240**(4857), 1335-9.

Javaherian, K., Langlois, A. J., LaRosa, G. J., Profy, A. T., Bolognesi, D. P., Herlihy, W. C., Putney, S. D., and Matthews, T. J. (1990). Broadly neutralizing antibodies elicited by the hypervariable neutralizing determinant of HIV-1. *Science* **250**(4987), 1590-3.

Javaherian, K., Langlois, A. J., McDanal, C., Ross, K. L., Eckler, L. I., Jellis, C. L., Profy, A. T., Rusche, J. R., Bolognesi, D. P., Putney, S. D., and et al. (1989). Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *Proc Natl Acad Sci U S A* **86**(17), 6768-72.

Jeffries, D. J. (1989). Targets for antiviral therapy of human immunodeficiency virus infection. *J Infect* **18 Suppl 1**, 5-13.

Jeffs, S. A., Goriup, S., Kebble, B., Crane, D., Bolgiano, B., Sattentau, Q., Jones, S., and Holmes, H. (2004). Expression and characterisation of recombinant oligomeric envelope glycoproteins derived from primary isolates of HIV-1. *Vaccine* **22**(8), 1032-46.

Jeffs, S. A., Goriup, S., Stacey, G., Yuen, C. T., and Holmes, H. (2006). Comparative analysis of HIV-1 recombinant envelope glycoproteins from different culture systems. *Appl Microbiol Biotechnol* **72**(2), 279-90.

Jeffs, S. A., McKeating, J., Lewis, S., Craft, H., Biram, D., Stephens, P. E., and Brady, R. L. (1996). Antigenicity of truncated forms of the human immunodeficiency virus type 1 envelope glycoprotein. *J Gen Virol* **77 (Pt 7)**, 1403-10.

Jeffs, S. A., Shotton, C., Balfe, P., and McKeating, J. A. (2002). Truncated gp120 envelope glycoprotein of human immunodeficiency virus 1 elicits a broadly reactive neutralizing immune response. *J Gen Virol* **83**(Pt 11), 2723-32.

Jiang, S., He, Y., and Liu, S. (2005). SARS vaccine development. *Emerg Infect Dis* **11**(7), 1016-20.

Jolly, C., and Sattentau, Q. J. (2005). Human immunodeficiency virus type 1 virological synapse formation in T cells requires lipid raft integrity. *J Virol* **79**(18), 12088-94.

Julg, B., and Goebel, F. D. (2005). HIV genetic diversity: any implications for drug resistance? *Infection* **33**(4), 299-301.

Kalyanaraman, V. S., Rausch, D. M., Osborne, J., Padgett, M., Hwang, K. M., Lifson, J. D., and Eiden, L. E. (1990). Evidence by peptide mapping that the region CD4(81-92) is involved in gp120/CD4 interaction leading to HIV infection and HIV-induced syncytium formation. *J Immunol* **145**(12), 4072-8.

Kanbe, K., Shimizu, N., Soda, Y., Takagishi, K., and Hoshino, H. (1999). A CXC chemokine receptor, CXCR5/BLR1, is a novel and specific coreceptor for human immunodeficiency virus type 2. *Virology* **265**(2), 264-73.

Kaneko, H., Hishikawa, T., Sekigawa, I., Hashimoto, H., Okumura, K., and Kaneko, Y. (1997a). Role of tumour necrosis factor-alpha (TNF-alpha) in the induction of HIV-1 gp120-mediated CD4+ T cell anergy. *Clin Exp Immunol* **109**(1), 41-6.

Kaneko, H., Neoh, L. P., Takeda, N., Akimoto, H., Hishikawa, T., Hashimoto, H., Hirose, S., Karaki, S., Takiguchi, M., Nakauchi, H., Kaneko, Y., Yamamoto, N., and Sekigawa, I. (1997b). Human immunodeficiency virus type 2 envelope glycoprotein binds to CD8 as well as to CD4 molecules on human T cells. *J Virol* **71**(11), 8918-22.

Kang, C. Y. (1997). Expression of human immunodeficiency virus genes using baculovirus expression system. *Mol Biotechnol* **8**(2), 173-87.

Kanki, P., M'Boup, S., Marlink, R., Travers, K., Hsieh, C. C., Gueye, A., Boye, C., Sankale, J. L., Donnelly, C., Leisenring, W., and et al. (1992). Prevalence and risk determinants of human immunodeficiency virus type 2 (HIV-2) and human immunodeficiency virus type 1 (HIV-1) in west African female prostitutes. *Am J Epidemiol* **136**(7), 895-907.

Kanki, P. J., Travers, K. U., S, M. B., Hsieh, C. C., Marlink, R. G., Gueye, N. A., Siby, T., Thior, I., Hernandez-Avila, M., Sankale, J. L., and et al. (1994). Slower heterosexual spread of HIV-2 than HIV-1. *Lancet* **343**(8903), 943-6.

Kedzierska, K., and Crowe, S. M. (2001). Cytokines and HIV-1: interactions and clinical implications. *Antivir Chem Chemother* **12**(3), 133-50.

Keefer, M. C., Graham, B. S., Belshe, R. B., Schwartz, D., Corey, L., Bolognesi, D. P., Stablein, D. M., Montefiori, D. C., McElrath, M. J., Clements, M. L., and et al. (1994). Studies of high doses of a human immunodeficiency virus type 1 recombinant glycoprotein 160 candidate vaccine in HIV type 1-seronegative humans. The AIDS Vaccine Clinical Trials Network. *AIDS Res Hum Retroviruses* **10**(12), 1713-23.

Kent, K. A., Gritz, L., Stallard, G., Cranage, M. P., Collignon, C., Thiriart, C., Corcoran, T., Silvera, P., and Stott, E. J. (1991). Production and of monoclonal antibodies to simian immunodeficiency virus envelope glycoproteins. *AIDS* **5**(7), 829-36.

Kent, K. A., Rud, E., Corcoran, T., Powell, C., Thiriart, C., Collignon, C., and Stott, E. J. (1992). Identification of two neutralizing and 8 non-neutralizing epitopes on simian immunodeficiency virus envelope using monoclonal antibodies. *AIDS Res Hum Retroviruses* **8**(6), 1147-51.

Kerkau, T., Bacik, I., Bennink, J. R., Yewdell, J. W., Hunig, T., Schimpl, A., and Schubert, U. (1997). The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step in the biosynthesis of major histocompatibility complex (MHC) class I molecules. *J Exp Med* **185**(7), 1295-305.

Kewalramani, V. N., and Emerman, M. (1996). Vpx association with mature core structures of HIV-2. *Virology* **218**(1), 159-68.

Kilby, J. M., and Eron, J. J. (2003). Novel therapies based on mechanisms of HIV-1 cell entry. *N Engl J Med* **348**(22), 2228-38.

Kim, M., Chen, B., Hussey, R. E., Chishti, Y., Montefiori, D., Hoxie, J. A., Byron, O., Campbell, G., Harrison, S. C., and Reinherz, E. L. (2001). The stoichiometry of trimeric SIV glycoprotein interaction with CD4 differs from that of anti-envelope antibody Fab fragments. *J Biol Chem* **276**(46), 42667-76.

Kinloch-de Loes, S., de Saussure, P., Saurat, J. H., Stalder, H., Hirschel, B., and Perrin, L. H. (1993). Symptomatic primary infection due to human immunodeficiency virus type 1: review of 31 cases. *Clin Infect Dis* **17**(1), 59-65.

Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J. C., and Montagnier, L. (1984). T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* **312**(5996), 767-8.

Klein, M. R., van Baalen, C. A., Holwerda, A. M., Kerkhof Garde, S. R., Bende, R. J., Keet, I. P., Eeftinck-Schattenkerk, J. K., Osterhaus, A. D., Schuitemaker, H., and Miedema, F. (1995). Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J Exp Med* **181**(4), 1365-72.

Klein, M. R., van der Burg, S. H., Hovenkamp, E., Holwerda, A. M., Drijfhout, J. W., Melief, C. J., and Miedema, F. (1998). Characterization of HLA-B57-restricted human immunodeficiency virus type 1 Gag- and RT-specific cytotoxic T lymphocyte responses. *J Gen Virol* **79 (Pt 9)**, 2191-201.

Kliger, Y., Gallo, S. A., Peisajovich, S. G., Munoz-Barroso, I., Avkin, S., Blumenthal, R., and Shai, Y. (2001). Mode of action of an antiviral peptide from HIV-1. Inhibition at a post-lipid mixing stage. *J Biol Chem* **276**(2), 1391-7.

Kolchinsky, P., Kiprilov, E., Bartley, P., Rubinstein, R., and Sodroski, J. (2001). Loss of a single N-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops. *J Virol* **75**(7), 3435-43.

Kolchinsky, P., Kiprilov, E., and Sodroski, J. (2001). Increased neutralization sensitivity of CD4-independent human immunodeficiency virus variants. *J Virol* **75**(5), 2041-50.

Kolchinsky, P., Mirzabekov, T., Farzan, M., Kiprilov, E., Cayabyab, M., Mooney, L. J., Choe, H., and Sodroski, J. (1999). Adaptation of a CCR5-using, primary human immunodeficiency virus type 1 isolate for CD4-independent replication. *J Virol* **73**(10), 8120-6.

Korba, B. E., and Milman, G. (1991). A cell culture assay for compounds which inhibit hepatitis B virus replication. *Antiviral Res* **15**(3), 217-28.

Korber, B., Muldoon, M., Theiler, J., Gao, F., Gupta, R., Lapedes, A., Hahn, B. H., Wolinsky, S., and Bhattacharya, T. (2000). Timing the ancestor of the HIV-1 pandemic strains. *Science* **288**(5472), 1789-96.

Kottilil, S., Chun, T. W., Moir, S., Liu, S., McLaughlin, M., Hallahan, C. W., Maldarelli, F., Corey, L., and Fauci, A. S. (2003). Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function. *J Infect Dis* **187**(7), 1038-45.

Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C., and Ho, D. D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* **68**(7), 4650-5.

Kowalski, M., Potz, J., Basiripour, L., Dorfman, T., Goh, W. C., Terwilliger, E., Dayton, A., Rosen, C., Haseltine, W., and Sodroski, J. (1987). Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science* **237**(4820), 1351-5.

Kozak, S. L., Platt, E. J., Madani, N., Ferro, F. E., Jr., Peden, K., and Kabat, D. (1997). CD4, CXCR-4, and CCR-5 dependencies for infections by primary patient and laboratory-adapted isolates of human immunodeficiency virus type 1. *J Virol* **71**(2), 873-82.

Kozlov, M. M., Leikin, S. L., Chernomordik, L. V., Markin, V. S., and Chizmadzhev, Y. A. (1989). Stalk mechanism of vesicle fusion. Intermixing of aqueous contents. *Eur Biophys J* **17**(3), 121-9.

Kozlovsky, Y., and Kozlov, M. M. (2002). Stalk model of membrane fusion: solution of energy crisis. *Biophys J* **82**(2), 882-95.

Kuhmann, S. E., Platt, E. J., Kozak, S. L., and Kabat, D. (2000). Cooperation of multiple CCR5 coreceptors is required for infections by human immunodeficiency virus type 1. *J Virol* **74**(15), 7005-15.

Kulkarni, S., Thakar, M., Rodrigues, J., and Banerjee, K. (1992). HIV-2 antibodies in serum samples from Maharashtra state. *Indian J Med Res* **95**, 213-5.

Kwong, P. D., Doyle, M. L., Casper, D. J., Cicala, C., Leavitt, S. A., Majeed, S., Steenbeke, T. D., Venturi, M., Chaiken, I., Fung, M., Katinger, H., Parren, P. W., Robinson, J., Van Ryk, D., Wang, L., Burton, D. R., Freire, E., Wyatt, R., Sodroski, J., Hendrickson, W. A., and Arthos, J. (2002). HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* **420**(6916), 678-82.

Kwong, P. D., Wyatt, R., Majeed, S., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (2000a). Structures of the HIV-1 gp120 Envelope Glycoproteins from Laboratory-Adapted and Primary Isolates. *Structure* **8**, 1329-1339.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648-659.

Kwong, P. D., Wyatt, R., Sattentau, Q. J., Sodroski, J., and Hendrickson, W. A. (2000b). Oligomeric Modeling and Electrostatic Analysis of the gp120 Envelope Glycoprotein of Human Immunodeficiency Virus. *Journal of Virology* **74**(4), 1961-1972.

LaBranche, C. C., Hoffman, T. L., Romano, J., Haggarty, B. S., Edwards, T. G., Matthews, T. J., Doms, R. W., and Hoxie, J. A. (1999). Determinants of CD4 independence for a human immunodeficiency virus type 1 variant map outside regions required for coreceptor specificity. *J Virol* **73**(12), 10310-9.

Labrosse, B., Treboute, C., Brelot, A., and Alizon, M. (2001). Cooperation of the V1/V2 and V3 domains of human immunodeficiency virus type 1 gp120 for interaction with the CXCR4 receptor. *J Virol* **75**(12), 5457-64.

Lamb, R. A., and Pinto, L. H. (1997). Do Vpu and Vpr of human immunodeficiency virus type 1 and NB of influenza B virus have ion channel activities in the viral life cycles? *Virology* **229**(1), 1-11.

Langley, C. L., Benga-De, E., Critchlow, C. W., Ndoye, I., Mbengue-Ly, M. D., Kuypers, J., Woto-Gaye, G., Mboup, S., Bergeron, C., Holmes, K. K., and Kiviat, N. B. (1996). HIV-1, HIV-2, human papillomavirus infection and cervical neoplasia in high-risk African women. *AIDS* **10**(4), 413-7.

Lasky, L. A., Groopman, J. E., Fennie, C. W., Benz, P. M., Capon, D. J., Dowbenko, D. J., Nakamura, G. R., Nunes, W. M., Renz, M. E., and Berman, P. W. (1986). Neutralization of the AIDS retrovirus by antibodies to a recombinant envelope glycoprotein. *Science* **233**(4760), 209-12.

Lasky, L. A., Nakamura, G., Smith, D. H., Fennie, C., Shimasaki, C., Patzer, E., Berman, P., Gregory, T., and Capon, D. J. (1987). Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. *Cell* **50**(6), 975-85.

Layne, S. P., Merges, M. J., Dembo, M., Spouge, J. L., and Nara, P. L. (1990). HIV requires multiple gp120 molecules for CD4-mediated infection. *Nature* **346**(6281), 277-9.

Le Grand, R., Vogt, G., Chapel, A., and Dormont, D. (1991). Antibody-dependent enhancement and neutralization pattern of sera from SIV-infected or HIV-2-vaccinated rhesus monkeys. *J Med Primatol* **20**(4), 172-6.

Le Jan, C., Bellaton, C., Greenland, T., and Mornex, J. F. (2005). Mammary transmission of caprine arthritis encephalitis virus: a 3D model for in vitro study. *Reprod Nutr Dev* **45**(4), 513-23.

Lee, W. R., Syu, W. J., Du, B., Matsuda, M., Tan, S., Wolf, A., Essex, M., and Lee, T. H. (1992). Nonrandom distribution of gp120 N-linked glycosylation sites important for infectivity of human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **89**(6), 2213-7.

Lehner, T. (2003). Innate and adaptive mucosal immunity in protection against HIV infection. *Vaccine* **21 Suppl 2**, S68-76.

Leitner, T., Kumar, S., and Albert, J. (1997). Tempo and mode of nucleotide substitutions in gag and env gene fragments in human immunodeficiency virus type 1 populations with a known transmission history. *J Virol* **71**(6), 4761-70.

Lemey, P., Pybus, O. G., Wang, B., Saksena, N. K., Salemi, M., and Vandamme, A. M. (2003). Tracing the origin and history of the HIV-2 epidemic. *Proc Natl Acad Sci U S A* **100**(11), 6588-92.

Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N., and Gregory, T. J. (1990). Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J Biol Chem* **265**(18), 10373-82.

Letarov, A. V., Londer, Y. Y., Boudko, S. P., and Mesyanzhinov, V. V. (1999). The carboxy-terminal domain initiates trimerization of bacteriophage T4 fibritin. *Biochemistry (Mosc)* **64**(7), 817-23.

Levy, J. A. (1998). "HIV and the Pathogenesis of AIDS." Second Edition ed. ASM Press, Washington D.C.

Levy, J. A., Hoffman, A. D., Kramer, S. M., Landis, J. A., Shimabukuro, J. M., and Oshiro, L. S. (1984). Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* **225**(4664), 840-2.

Levy, J. A., Mackewicz, C. E., and Barker, E. (1996). Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8+ T cells. *Immunol Today* **17**(5), 217-24.

Lewis, J. S., 2nd, Terriff, C. M., Coulston, D. R., and Garrison, M. W. (1997). Protease inhibitors: a therapeutic breakthrough for the treatment of patients with human immunodeficiency virus. *Clin Ther* **19**(2), 187-214.

Li, L., Li, H. S., Pauza, C. D., Bukrinsky, M., and Zhao, R. Y. (2005a). Roles of HIV-1 auxiliary proteins in viral pathogenesis and host-pathogen interactions. *Cell Res* **15**(11-12), 923-34.

Li, Q., Duan, L., Estes, J. D., Ma, Z. M., Rourke, T., Wang, Y., Reilly, C., Carlis, J., Miller, C. J., and Haase, A. T. (2005b). Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* **434**(7037), 1148-52.

Li, W. H., Tanimura, M., and Sharp, P. M. (1988). Rates and dates of divergence between AIDS virus nucleotide sequences. *Mol Biol Evol* **5**(4), 313-30.

Li, Y., Bergeron, J. J., Luo, L., Ou, W. J., Thomas, D. Y., and Kang, C. Y. (1996). Effects of inefficient cleavage of the signal sequence of HIV-1 gp 120 on its association with calnexin, folding, and intracellular transport. *Proc Natl Acad Sci U S A* **93**(18), 9606-11.

Li, Y., Luo, L., Rasool, N., and Kang, C. Y. (1993). Glycosylation is necessary for the correct folding of human immunodeficiency virus gp120 in CD4 binding. *J Virol* **67**(1), 584-8.

Li, Y., Luo, L., Thomas, D. Y., and Kang, C. Y. (1994). Control of expression, glycosylation, and secretion of HIV-1 gp120 by homologous and heterologous signal sequences. *Virology* **204**(1), 266-78.

Li, Y., Luo, L., Thomas, D. Y., and Kang, C. Y. (2000). The HIV-1 Env protein signal sequence retards its cleavage and down-regulates the glycoprotein folding. *Virology* **272**(2), 417-28.

Li, Y., Svehla, K., Mathy, N. L., Voss, G., Mascola, J. R., and Wyatt, R. (2006). Characterization of antibody responses elicited by human immunodeficiency virus type 1 primary isolate trimeric and monomeric envelope glycoproteins in selected adjuvants. *J Virol* **80**(3), 1414-26.

Liao, F., Alkhatib, G., Peden, K. W., Sharma, G., Berger, E. A., and Farber, J. M. (1997). STRL33, A novel chemokine receptor-like protein, functions as a fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1. *J Exp Med* **185**(11), 2015-23.

Liedtke, S., Adamski, M., Geyer, R., Pfutzner, A., Rubsamen-Waigmann, H., and Geyer, H. (1994). Oligosaccharide profiles of HIV-2 external envelope glycoprotein: dependence on host cells and virus isolates. *Glycobiology* **4**(4), 477-84.

Liedtke, S., Geyer, R., and Geyer, H. (1997). Host-cell-specific glycosylation of HIV-2 envelope glycoprotein. *Glycoconjugate Journal* **14**, 785-793.

Lifson, J. D., Hwang, K. M., Nara, P. L., Fraser, B., Padgett, M., Dunlop, N. M., and Eiden, L. E. (1988). Synthetic CD4 peptide derivatives that inhibit HIV infection and cytopathicity. *Science* **241**(4866), 712-6.

Lineberger, J. E., Danzeisen, R., Hazuda, D. J., Simon, A. J., and Miller, M. D. (2002). Altering expression levels of human immunodeficiency virus type 1 gp120-gp41 affects efficiency but not kinetics of cell-cell fusion. *J Virol* **76**(7), 3522-33.

Lisse, I. M., Poulsen, A. G., Aaby, P., Knudsen, K., and Dias, F. (1996). Serial CD4 and CD8 T-lymphocyte counts and associated mortality in an HIV-2-infected population in Guinea-Bissau. *J Acquir Immune Defic Syndr Hum Retrovirol* **13**(4), 355-62.

Little, S. J., Daar, E. S., D'Aquila, R. T., Keiser, P. H., Connick, E., Whitcomb, J. M., Hellmann, N. S., Petropoulos, C. J., Sutton, L., Pitt, J. A., Rosenberg, E. S., Koup, R. A., Walker, B. D., and Richman, D. D. (1999). Reduced antiretroviral drug susceptibility among patients with primary HIV infection. *Jama* **282**(12), 1142-9.

Littman, D. R. (1987). The structure of the CD4 and CD8 genes. *Annu Rev Immunol* **5**, 561-84.

Liu, H., Wu, X., Newman, M., Shaw, G. M., Hahn, B. H., and Kappes, J. C. (1995). The Vif protein of human and simian immunodeficiency viruses is packaged into virions and associates with viral core structures. *J Virol* **69**(12), 7630-8.

Liu, H. Y., Soda, Y., Shimizu, N., Haraguchi, Y., Jinno, A., Takeuchi, Y., and Hoshino, H. (2000). CD4-Dependent and CD4-independent utilization of coreceptors by human immunodeficiency viruses type 2 and simian immunodeficiency viruses. *Virology* **278**(1), 276-88.

Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A., and Landau, N. R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**(3), 367-77.

Locher, C. P., Witt, S. A., Ashlock, B. M., and Levy, J. A. (2002). Enhancement of antibody responses to an HIV-2 DNA envelope vaccine using an expression vector containing a constitutive transport element. *DNA Cell Biol* **21**(8), 581-6.

Locher, C. P., Witt, S. A., Ashlock, B. M., and Levy, J. A. (2004). Evaluation of genetic immunization adjuvants to improve the effectiveness of a human immunodeficiency virus type 2 (HIV-2) envelope DNA vaccine. *DNA Cell Biol* **23**(2), 107-10.

Louwagie, J., McCutchan, F. E., Peeters, M., Brennan, T. P., Sanders-Buell, E., Eddy, G. A., van der Groen, G., Fransen, K., Gershy-Damet, G. M., Deleys, R., and et al. (1993). Phylogenetic analysis of gag genes from 70 international HIV-1 isolates provides evidence for multiple genotypes. *AIDS* **7**(6), 769-80.

Lu, M., Blacklow, S. C., and Kim, P. S. (1995). A trimeric structural domain of the HIV-1 transmembrane glycoprotein. *Nat Struct Biol* **2**(12), 1075-82.

Lu, Y. L., Spearman, P., and Ratner, L. (1993). Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. *J Virol* **67**(11), 6542-50.

Lucas, S. B., Hounnou, A., Peacock, C., Beaumel, A., Djomand, G., N'Gbichi, J. M., Yeboue, K., Honde, M., Diomande, M., Giordano, C., and et al. (1993). The mortality and pathology of HIV infection in a west African city. *AIDS* **7**(12), 1569-79.

Ly, A., and Stamatatos, L. (2000). V2 loop glycosylation of the human immunodeficiency virus type 1 SF162 envelope facilitates interaction of this protein with CD4 and CCR5 receptors and protects the virus from neutralization by anti-V3 loop and anti-CD4 binding site antibodies. *J Virol* **74**(15), 6769-76.

Maddon, P. J., Littman, D. R., Godfrey, M., Maddon, D. E., Chess, L., and Axel, R. (1985). The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. *Cell* **42**(1), 93-104.

Maddon, P. J., Molineaux, S. M., Maddon, D. E., Zimmerman, K. A., Godfrey, M., Alt, F. W., Chess, L., and Axel, R. (1987). Structure and expression of the human and mouse T4 genes. *Proc Natl Acad Sci U S A* **84**(24), 9155-9.

Mahmood, N., and Hay, A. J. (1992). An ELISA utilizing immobilised snowdrop lectin GNA for the detection of envelope glycoproteins of HIV and SIV. *J Immunol Methods* **151**(1-2), 9-13.

Majeed, S., Ofek, G., Belachew, A., Huang, C. C., Zhou, T., and Kwong, P. D. (2003). Enhancing protein crystallization through precipitant synergy. *Structure (Camb)* **11**(9), 1061-70.

Malenbaum, S. E., Yang, D., Cavacini, L., Posner, M., Robinson, J., and Cheng-Mayer, C. (2000). The N-terminal V3 loop glycan modulates the interaction of clade A and B human immunodeficiency virus type 1 envelopes with CD4 and chemokine receptors. *J Virol* **74**(23), 11008-16.

Malim, M. H., Hauber, J., Le, S. Y., Maizel, J. V., and Cullen, B. R. (1989). The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature* **338**(6212), 254-7.

Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., and Trono, D. (2003). Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* **424**(6944), 99-103.

Mansky, L. M. (1996). The mutation rate of human immunodeficiency virus type 1 is influenced by the vpr gene. *Virology* **222**(2), 391-400.

Marciani, D. J., and Papamatheakis, J. D. (1978). Anomalous behavior of the major avian myeloblastosis virus glycoprotein in the presence of sodium dodecyl sulfate. *J Virol* **26**(3), 825-7.

Mariani, R., Chen, D., Schrofelbauer, B., Navarro, F., Konig, R., Bollman, B., Munk, C., Nymark-McMahon, H., and Landau, N. R. (2003). Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* **114**(1), 21-31.

Marin, M., Rose, K. M., Kozak, S. L., and Kabat, D. (2003). HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. *Nat Med* **9**(11), 1398-403.

Markin, V. S., and Albanesi, J. P. (2002). Membrane fusion: stalk model revisited. *Biophys J* **82**(2), 693-712.

Markin, V. S., Kozlov, M. M., and Borovjagin, V. L. (1984). On the theory of membrane fusion. The stalk mechanism. *Gen Physiol Biophys* **3**(5), 361-77.

Markosyan, R. M., Cohen, F. S., and Melikyan, G. B. (2003). HIV-1 envelope proteins complete their folding into six-helix bundles immediately after fusion pore formation. *Mol Biol Cell* **14**(3), 926-38.

Marlink, R. (1996). Lessons from the second AIDS virus, HIV-2. *AIDS* **10**, 689-699.

Marlink, R., Kanki, P., Thior, I., Travers, K., Eisen, G., Siby, T., Traore, I., Hsieh, C. C., Dia, M. C., Gueye, E. H., and et al. (1994). Reduced rate of disease development after HIV-2 infection as compared to HIV-1. *Science* **265**(5178), 1587-90.

Marx, P. A., Li, Y., Lerche, N. W., Sutjipto, S., Gettie, A., Yee, J. A., Brotman, B. H., Prince, A. M., Hanson, A., Webster, R. G., and et al. (1991). Isolation of a simian immunodeficiency virus related to human immunodeficiency virus type 2 from a west African pet sooty mangabey. *J Virol* **65**(8), 4480-5.

Mascola, J. R., Snyder, S. W., Weislow, O. S., Belay, S. M., Belshe, R. B., Schwartz, D. H., Clements, M. L., Dolin, R., Graham, B. S., Gorse, G. J., Keefer, M. C., McElrath, M. J., Walker, M. C., Wagner, K. F., McNeil, J. G., McCutchan, F. E., and Burke, D. S. (1996). Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. The National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group. *J Infect Dis* **173**(2), 340-8.

Mascola, J. R., Stiegler, G., VanCott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., Beary, H., Hayes, D., Frankel, S. S., Birx, D. L., and Lewis, M. G. (2000). Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* **6**(2), 207-10.

Masur, H., Michelis, M. A., Greene, J. B., Onorato, I., Stouwe, R. A., Holzman, R. S., Wormser, G., Brettman, L., Lange, M., Murray, H. W., and Cunningham-Rundles, S. (1981). An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. *N Engl J Med* **305**(24), 1431-8.

Masur, H., Ognibene, F. P., Yarchoan, R., Shelhamer, J. H., Baird, B. F., Travis, W., Suffredini, A. F., Deyton, L., Kovacs, J. A., Falloon, J., and et al. (1989). CD4 counts as predictors of opportunistic pneumonias in human immunodeficiency virus (HIV) infection. *Ann Intern Med* **111**(3), 223-31.

Matheron, S., Mendoza-Sassi, G., Simon, F., Olivares, R., Coulaud, J. P., and Brun-Vezinet, F. (1997). HIV-1 and HIV-2 AIDS in African patients living in Paris. *AIDS* **11**(7), 934-6.

Matheron, S., Pueyo, S., Damond, F., Simon, F., Lepretre, A., Campa, P., Salamon, R., Chene, G., and Brun-Vezinet, F. (2003). Factors associated with clinical progression in HIV-2 infected-patients: the French ANRS cohort. *AIDS* **17**(18), 2593-601.

Matsushita, S., Matsumi, S., Yoshimura, K., Morikita, T., Murakami, T., and Takatsuki, K. (1995). Neutralizing monoclonal antibodies against human immunodeficiency virus type 2 gp120. *J Virol* **69**(6), 3333-40.

Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M., and Roederer, M. (2005). Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* **434**(7037), 1093-7.

Matthews, T. J. (1994). Dilemma of neutralization resistance of HIV-1 field isolates and vaccine development. *AIDS Res Hum Retroviruses* **10**(6), 631-2.

McGaughey, G. B., Citron, M., Danzeisen, R. C., Freidinger, R. M., Garsky, V. M., Hurni, W. M., Joyce, J. G., Liang, X., Miller, M., Shiver, J., and Bogusky, M. J. (2003). HIV-1 vaccine development: constrained peptide immunogens show improved binding to the anti-HIV-1 gp41 MAb. *Biochemistry* **42**(11), 3214-23.

McKay, P. F., Barouch, D. H., Schmitz, J. E., Veazey, R. S., Gorgone, D. A., Lifton, M. A., Williams, K. C., and Letvin, N. L. (2003). Global dysfunction of CD4 T-lymphocyte cytokine expression in simian-human immunodeficiency virus/SIV-infected monkeys is prevented by vaccination. *J Virol* **77**(8), 4695-702.

McKnight, A., Dittmar, M. T., Moniz-Periera, J., Ariyoshi, K., Reeves, J. D., Hibbitts, S., Whitby, D., Aarons, E., Proudfoot, A. E. I., Whittle, H., and Clapham, P. R. (1998). A Broad range of chemokine receptors are used by Primary Isolates of Human Immunodeficiency Virus Type 2 as coreceptors with CD4. *Journal of Virology* **72**(5), 4065-4071.

McKnight, A., Shotton, C., Cordell, J., Jones, I., Simmons, G., and Clapham, P. R. (1996). Location, exposure, and conservation of neutralizing and nonneutralizing epitopes on human immunodeficiency virus type 2 SU glycoprotein. *J Virol* **70**(7), 4598-606.

McNeil, A. J., Yap, P. L., Gore, S. M., Brettle, R. P., McColl, M., Wyld, R., Davidson, S., Weightman, R., Richardson, A. M., and Robertson, J. R. (1996). Association of HLA types A1-B8-DR3 and B27 with rapid and slow progression of HIV disease. *Qjm* **89**(3), 177-85.

Mehandru, S., Poles, M. A., Tenner-Racz, K., Horowitz, A., Hurley, A., Hogan, C., Boden, D., Racz, P., and Markowitz, M. (2004). Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* **200**(6), 761-70.

Mehle, A., Strack, B., Ancuta, P., Zhang, C., McPike, M., and Gabuzda, D. (2004). Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. *J Biol Chem* **279**(9), 7792-8.

Melikyan, G. B., Markosyan, R. M., Hemmati, H., Delmedico, M. K., Lambert, D. M., and Cohen, F. S. (2000). Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. *J Cell Biol* **151**(2), 413-23.

Mellors, J. W., Kingsley, L. A., Rinaldo, C. R., Jr., Todd, J. A., Hoo, B. S., Kokka, R. P., and Gupta, P. (1995). Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. *Ann Intern Med* **122**(8), 573-9.

Mellors, J. W., Munoz, A., Giorgi, J. V., Margolick, J. B., Tassoni, C. J., Gupta, P., Kingsley, L. A., Todd, J. A., Saah, A. J., Detels, R., Phair, J. P., and Rinaldo, C. R., Jr. (1997). Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. *Ann Intern Med* **126**(12), 946-54.

Mellors, J. W., Rinaldo, C. R., Jr., Gupta, P., White, R. M., Todd, J. A., and Kingsley, L. A. (1996). Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science* **272**(5265), 1167-70.

Meyerhans, A., Cheynier, R., Albert, J., Seth, M., Kwok, S., Sninsky, J., Morfeldt-Manson, L., Asjo, B., and Wain-Hobson, S. (1989). Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. *Cell* **58**(5), 901-10.

Miller, M. D., Farnet, C. M., and Bushman, F. D. (1997). Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. *J Virol* **71**(7), 5382-90.

Mimura, Y., Lund, J., Church, S., Dong, S., Li, J., Goodall, M., and Jefferis, R. (2001). Butyrate increases production of human chimeric IgG in CHO-K1 cells whilst maintaining function and glycoform profile. *J Immunol Methods* **247**(1-2), 205-16.

Mitsuya, H., Weinhold, K. J., Furman, P. A., St Clair, M. H., Lehrman, S. N., Gallo, R. C., Bolognesi, D., Barry, D. W., and Broder, S. (1985). 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. *Proc Natl Acad Sci U S A* **82**(20), 7096-100.

Mizukami, T., Fuerst, T. R., Berger, E. A., and Moss, B. (1988). Binding region for human immunodeficiency virus (HIV) and epitopes for HIV-blocking monoclonal antibodies of the CD4 molecule defined by site-directed mutagenesis. *Proc Natl Acad Sci U S A* **85**(23), 9273-7.

Mizuochi, T., Spellman, M. W., Larkin, M., Solomon, J., Basa, L. J., and Feizi, T. (1988a). Carbohydrate structures of the human-immunodeficiency-virus (HIV) recombinant envelope glycoprotein gp120 produced in Chinese-hamster ovary cells. *Biochem J* **254**(2), 599-603.

Mizuochi, T., Spellman, M. W., Larkin, M., Solomon, J., Basa, L. J., and Feizi, T. (1988b). Structural characterization by chromatographic profiling of the oligosaccharides of human immunodeficiency virus (HIV) recombinant envelope glycoprotein gp120 produced in Chinese hamster ovary cells. *Biomed Chromatogr* **2**(6), 260-70.

Modrow, S., Hahn, B. H., Shaw, G. M., Gallo, R. C., Wong-Staal, F., and Wolf, H. (1987). Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. *J Virol* **61**(2), 570-8.

Montal, M. (2003). Structure-function correlates of Vpu, a membrane protein of HIV-1. *FEBS Lett* **552**(1), 47-53.

Moog, C., Fleury, H. J., Pellegrin, I., Kirn, A., and Aubertin, A. M. (1997). Autologous and heterologous neutralizing antibody responses following initial seroconversion in human immunodeficiency virus type 1-infected individuals. *J Virol* **71**(5), 3734-41.

Moore, J. P. (1990). Simple methods for monitoring HIV-1 and HIV-2 gp120 binding to soluble CD4 by enzyme-linked immunosorbent assay: HIV-2 has a 25-fold lower affinity than HIV-1 for soluble CD4. *AIDS* **4**(4), 297-305.

Moore, J. P., Burkly, L. C., Connor, R. I., Cao, Y., Tizard, R., Ho, D. D., and Fisher, R. A. (1993). Adaptation of two primary human immunodeficiency virus type 1 isolates to growth in transformed T cell lines correlates with alterations in the responses of their envelope glycoproteins to soluble CD4. *AIDS Res Hum Retroviruses* **9**(6), 529-39.

Moore, J. P., and Burton, D. R. (2004). Urgently needed: a filter for the HIV-1 vaccine pipeline. *Nat Med* **10**(8), 769-71.

Moore, J. P., McKeating, J. A., Jones, I. M., Stephens, P. E., Clements, G., Thomson, S., and Weiss, R. A. (1990a). Characterization of recombinant gp120 and gp160 from HIV-1: binding to monoclonal antibodies and soluble CD4. *AIDS* **4**(4), 307-15.

Moore, J. P., McKeating, J. A., Weiss, R. A., and Sattentau, Q. J. (1990b). Dissociation of gp120 from HIV-1 virions induced by soluble CD4. *Science* **250**(4984), 1139-42.

Moore, J. P., and Sodroski, J. (1996). Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein. *J Virol* **70**(3), 1863-72.

Moore, J. P., Trkola, A., and Dragic, T. (1997). Co-receptors for HIV-1 entry. *Curr Opin Immunol* **9**(4), 551-62.

Morgan, G., Wilkins, H. A., Pepin, J., Jobe, O., Brewster, D., and Whittle, H. (1990). AIDS following mother-to-child transmission of HIV-2. *AIDS* **4**(9), 879-82.

Morikawa, Y., Moore, J. P., Wilkinson, A. J., and Jones, I. M. (1991). Reduction in CD4 binding affinity associated with removal of a single glycosylation site in the external glycoprotein of HIV-2. *Virology* **180**(2), 853-6.

Morner, A., Thomas, J. A., Bjorling, E., Munson, P. J., Lucas, S. B., and McKnight, A. (2003). Productive HIV-2 infection in the brain is restricted to macrophages/microglia. *Aids* **17**(10), 1451-1455.

Morris, K. F., Gao, X., and Wong, T. C. (2004). The interactions of the HIV gp41 fusion peptides with zwitterionic membrane mimics determined by NMR spectroscopy. *Biochim Biophys Acta* **1667**(1), 67-81.

Most, J., Spotl, L., Sarcletti, M., Vogetseder, W., Dierich, M. P., and Zangerle, R. (1994). Differences in heterosexual transmission of HIV-1 and HIV-2. *Lancet* **344**(8936), 1572-3.

Mota-Miranda, A., Gomes, H., Marques, R., Serrao, R., Lourenco, H., Santos-Ferreira, O., and Lecour, H. (1995). HIV-2 infection with a long asymptomatic period. *J Infect* **31**(2), 163-4.

Moulard, M., Hallenberger, S., Garten, W., and Klenk, H. D. (1999). Processing and routage of HIV glycoproteins by furin to the cell surface. *Virus Res* **60**(1), 55-65.

Mulanga-Kabeya, C., Nzilambi, N., Edidi, B., Minlangu, M., Tshimpaka, T., Kambembo, L., Atibu, L., Mama, N., Ilunga, W., Sema, H., Tshimanga, K., Bongo, B., Peeters, M., and Delaporte, E. (1998). Evidence of stable HIV seroprevalences in selected populations in the Democratic Republic of the Congo. *AIDS* **12**(8), 905-10.

Muller, M. C., and Barre-Sinoussi, F. (2003). SIVagm: genetic and biological features associated with replication. *Front Biosci* **8**, d1170-85.

Mulligan, M. J., Ritter, G. D., Jr., Chaikin, M. A., Yamshchikov, G. V., Kumar, P., Hahn, B. H., Sweet, R. W., and Compans, R. W. (1992a). Human immunodeficiency virus type 2 envelope glycoprotein: differential CD4 interactions of soluble gp120 versus the assembled envelope complex. *Virology* **187**(1), 233-41.

Mulligan, M. J., Yamshchikov, G. V., Ritter, G. D., Jr., Gao, F., Jin, M. J., Nail, C. D., Spies, C. P., Hahn, B. H., and Compans, R. W. (1992b). Cytoplasmic domain truncation enhances fusion activity by the exterior glycoprotein complex of human immunodeficiency virus type 2 in selected cell types. *J Virol* **66**(6), 3971-5.

Mullins, C., Eisen, G., Popper, S., Dieng Sarr, A., Sankale, J. L., Berger, J. J., Wright, S. B., Chang, H. R., Coste, G., Cooley, T. P., Rice, P., Skolnik, P. R., Sullivan, M., and Kanki, P. J. (2004). Highly active antiretroviral therapy and viral response in HIV type 2 infection. *Clin Infect Dis* **38**(12), 1771-9.

Mummidi, S., Ahuja, S. S., Gonzalez, E., Anderson, S. A., Santiago, E. N., Stephan, K. T., Craig, F. E., O'Connell, P., Tryon, V., Clark, R. A., Dolan, M. J., and Ahuja, S. K. (1998). Genealogy of the CCR5 locus and chemokine system gene variants associated with altered rates of HIV-1 disease progression. *Nat Med* **4**(7), 786-93.

Munoz-Barroso, I., Durell, S., Sakaguchi, K., Appella, E., and Blumenthal, R. (1998). Dilation of the human immunodeficiency virus-1 envelope glycoprotein fusion pore revealed by the inhibitory action of a synthetic peptide from gp41. *J Cell Biol* **140**(2), 315-23.

Murakami, T., Nakajima, T., Koyanagi, Y., Tachibana, K., Fujii, N., Tamamura, H., Yoshida, N., Waki, M., Matsumoto, A., Yoshie, O., Kishimoto, T., Yamamoto, N., and Nagasawa, T. (1997). A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. *J Exp Med* **186**(8), 1389-93.

Murphy, C. I., Lennick, M., Lehar, S. M., Beltz, G. A., and Young, E. (1990). Temporal expression of HIV-1 envelope proteins in baculovirus-infected insect cells: implications for glycosylation and CD4 binding. *Genet Anal Tech Appl* **7**(6), 160-71.

Murphy, E., Korber, B., Georges-Courbot, M. C., You, B., Pinter, A., Cook, D., Kieny, M. P., Georges, A., Mathiot, C., Barre-Sinoussi, F., and et al. (1993). Diversity of V3 region sequences of human immunodeficiency viruses type 1 from the central African Republic. *AIDS Res Hum Retroviruses* **9**(10), 997-1006.

Murphy, P. M. (2002). International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. *Pharmacol Rev* **54**(2), 227-9.

Murphy, P. M., Baggiolini, M., Charo, I. F., Hebert, C. A., Horuk, R., Matsushima, K., Miller, L. H., Oppenheim, J. J., and Power, C. A. (2000). International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* **52**(1), 145-76.

Muster, T., Steindl, F., Purtscher, M., Trkola, A., Klima, A., Himmler, G., Ruker, F., and Katinger, H. (1993). A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J Virol* **67**(11), 6642-7.

Myers, G. (1994). Tenth anniversary perspectives on AIDS. HIV: between past and future. *AIDS Res Hum Retroviruses* **10**(11), 1317-24.

Myszka, D. G., Sweet, R. W., Hensley, P., Brigham-Burke, M., Kwong, P. D., Hendrickson, W. A., Wyatt, R., Sodroski, J., and Doyle, M. L. (2000). Energetics of the HIV gp120-CD4 binding reaction. *Proc Natl Acad Sci U S A* **97**(16), 9026-31.

Nabatov, A. A., Pollakis, G., Linnemann, T., Kliphius, A., Chalaby, M. I., and Paxton, W. A. (2004). Intrapatient alterations in the human immunodeficiency virus type 1 gp120 V1V2 and V3 regions differentially modulate coreceptor usage, virus inhibition by CC/CXC chemokines, soluble CD4, and the b12 and 2G12 monoclonal antibodies. *J Virol* **78**(1), 524-30.

Nabel, G. J. (2005). Immunology. Close to the edge: neutralizing the HIV-1 envelope. *Science* **308**(5730), 1878-9.

References

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* **382**(6592), 635-8.

Nakashima, H., Masuda, M., Murakami, T., Koyanagi, Y., Matsumoto, A., Fujii, N., and Yamamoto, N. (1992). Anti-human immunodeficiency virus activity of a novel synthetic peptide, T22 ([Tyr-5,12, Lys-7]polyphemusin II): a possible inhibitor of virus-cell fusion. *Antimicrob Agents Chemother* **36**(6), 1249-55.

Nakowitsch, S., Quendler, H., Fekete, H., Kunert, R., Katinger, H., and Stiegler, G. (2005). HIV-1 mutants escaping neutralization by the human antibodies 2F5, 2G12, and 4E10: in vitro experiments versus clinical studies. *Aids* **19**(17), 1957-66.

Nandi, J., Kamat, H., Bhavalkar, V., and Banerjee, K. (1994). Detection of human immunodeficiency virus antibody among homosexual men from Bombay. *Sex Transm Dis* **21**(4), 235-6.

Nandwani, R., Shanson, D., and Coleman, J. C. (1994). Missed HIV-2 infection in a bisexual man. *Int J STD AIDS* **5**(2), 155.

Neoh, L. P., Akimoto, H., Kaneko, H., Hishikawa, T., Hashimoto, H., Hirose, S., Kaneko, Y., Yamamoto, N., and Sekigawa, I. (1997). The production of beta-chemokines induced by HIV-2 envelope glycoprotein. *AIDS* **11**(8), 1062-3.

Nguyen, L., Li, M., Chaowanachan, T., Hu, D. J., Vanichseni, S., Mock, P. A., van Griensven, F., Martin, M., Sangkum, U., Choopanya, K., Tappero, J. W., Lal, R. B., and Yang, C. (2004). CCR5 promoter human haplogroups associated with HIV-1 disease progression in Thai injection drug users. *AIDS* **18**(9), 1327-33.

Ni, J., Song, H., Wang, Y., Stamatos, N. M., and Wang, L. X. (2006). Toward a carbohydrate-based HIV-1 vaccine: synthesis and immunological studies of oligomannose-containing glycoconjugates. *Bioconjug Chem* **17**(2), 493-500.

Nieva, J. L., and Agirre, A. (2003). Are fusion peptides a good model to study viral cell fusion? *Biochim Biophys Acta* **1614**(1), 104-15.

Nieva, J. L., Nir, S., Muga, A., Goni, F. M., and Wilschut, J. (1994). Interaction of the HIV-1 fusion peptide with phospholipid vesicles: different structural requirements for fusion and leakage. *Biochemistry* **33**(11), 3201-9.

Niu, M. T., Stein, D. S., and Schnittman, S. M. (1993). Primary human immunodeficiency virus type 1 infection: review of pathogenesis and early treatment intervention in humans and animal retrovirus infections. *J Infect Dis* **168**(6), 1490-501.

Norrgren, H., Andersson, S., Biague, A. J., da Silva, Z. J., Dias, F., Naucler, A., and Biberfeld, G. (1999). Trends and interaction of HIV-1 and HIV-2 in Guinea-Bissau, west Africa: no protection of HIV-2 against HIV-1 infection. *AIDS* **13**(6), 701-7.

Norrgren, H., Andersson, S., Naucler, A., Dias, F., Johansson, I., and Biberfeld, G. (1995). HIV-1, HIV-2, HTLV-I/II and Treponema pallidum infections: incidence, prevalence, and HIV-2-associated mortality in an occupational cohort in Guinea-Bissau. *J Acquir Immune Defic Syndr Hum Retrovirol* **9**(4), 422-8.

Norrgren, H., Cardoso, A. N., da Silva, Z. J., Andersson, S., Dias, F., Biberfeld, G., and Naucler, A. (1997). Increased prevalence of HIV-2 infection in hospitalized patients with severe bacterial diseases in Guinea-Bissau. *Scand J Infect Dis* **29**(5), 453-9.

Nunn, A. J., Mulder, D. W., Kamali, A., Ruberantwari, A., Kengeya-Kayondo, J. F., and Whitworth, J. (1997). Mortality associated with HIV-1 infection over five years in a rural Ugandan population: cohort study. *Bmj* **315**(7111), 767-71.

O'Donovan, D., Ariyoshi, K., Milligan, P., Ota, M., Yamuah, L., Sarge-Njie, R., and Whittle, H. (2000). Maternal plasma viral RNA levels determine marked differences in mother-to-child transmission rates of HIV-1 and HIV-2 in The Gambia. MRC/Gambia Government/University College London Medical School working group on mother-child transmission of HIV. *AIDS* **14**(4), 441-8.

Ogert, R. A., Lee, M. K., Ross, W., Buckler-White, A., Martin, M. A., and Cho, M. W. (2001). N-linked glycosylation sites adjacent to and within the V1/V2 and the V3 loops of dualtropic human immunodeficiency virus type 1 isolate DH12 gp120 affect coreceptor usage and cellular tropism. *J Virol* **75**(13), 5998-6006.

Orloff, G. M., Kalish, M. L., Chiphangwi, J., Potts, K. E., Ou, C. Y., Schochetman, G., Dallabetta, G., Saah, A. I., and Miotti, P. G. (1993). V3 loops of HIV-1 specimens from pregnant women in Malawi uniformly lack a potential N-linked glycosylation site. *AIDS Res Hum Retroviruses* **9**(7), 705-6.

Oroszlan, S., and Luftig, R. B. (1990). Retroviral proteinases. *Curr Top Microbiol Immunol* **157**, 153-85.

Osborn, L., Kunkel, S., and Nabel, G. J. (1989). Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc Natl Acad Sci U S A* **86**(7), 2336-40.

Ostermeier, C., and Michel, H. (1997). Crystallization of membrane proteins. *Curr Opin Struct Biol* **7**(5), 697-701.

Ostrowski, M. A., Krakauer, D. C., Li, Y., Justement, S. J., Learn, G., Ehler, L. A., Stanley, S. K., Nowak, M., and Fauci, A. S. (1998). Effect of immune activation on the dynamics of human immunodeficiency virus replication and on the distribution of viral quasispecies. *J Virol* **72**(10), 7772-84.

Ostrowski, S. R., Gerstoft, J., Pedersen, B. K., and Ullum, H. (2003). Impaired production of cytokines is an independent predictor of mortality in HIV-1-infected patients. *AIDS* **17**(4), 521-30.

Ott, D. E., Coren, L. V., Kane, B. P., Busch, L. K., Johnson, D. G., Sowder, R. C., 2nd, Chertova, E. N., Arthur, L. O., and Henderson, L. E. (1996). Cytoskeletal proteins inside human immunodeficiency virus type 1 virions. *J Virol* **70**(11), 7734-43.

Otteken, A., Voss, G., and Hunsmann, G. (1993). Mapping of the human immunodeficiency virus type 2 envelope glycoprotein CD4 binding region and fusion domain with truncated proteins expressed by recombinant vaccinia viruses. *Virology* **194**(1), 37-43.

Owen, S. M., Ellenberger, D., Rayfield, M., Wiktor, S., Michel, P., Grieco, M. H., Gao, F., Hahn, B. H., and Lal, R. B. (1998). Genetically divergent strains of human immunodeficiency virus type 2 use multiple coreceptors for viral entry. *J Virol* **72**(7), 5425-32.

Owman, C., Garzino-Demo, A., Cocchi, F., Popovic, M., Sabirsh, A., and Gallo, R. C. (1998). The leukotriene B4 receptor functions as a novel type of coreceptor mediating entry of primary HIV-1 isolates into CD4-positive cells. *Proc Natl Acad Sci U S A* **95**(16), 9530-4.

Ozel, M., Pauli, G., and Gelderblom, H. R. (1988). The organization of the envelope projections on the surface of HIV. *Arch Virol* **100**(3-4), 255-66.

Padian, N. S., Shiboski, S. C., Glass, S. O., and Vittinghoff, E. (1997). Heterosexual transmission of human immunodeficiency virus (HIV) in northern California: results from a ten-year study. *Am J Epidemiol* **146**(4), 350-7.

Padua, E., Jenkins, A., Brown, S., Bootman, J., Paixao, M. T., Almond, N., and Berry, N. (2003). Natural variation of the nef gene in human immunodeficiency virus type 2 infections in Portugal. *J Gen Virol* **84**(Pt 5), 1287-99.

Pancera, M., Lebowitz, J., Schon, A., Zhu, P., Freire, E., Kwong, P. D., Roux, K. H., Sodroski, J., and Wyatt, R. (2005). Soluble mimetics of human immunodeficiency virus type 1 viral spikes produced by replacement of the native trimerization domain with a heterologous trimerization motif: characterization and ligand binding analysis. *J Virol* **79**(15), 9954-69.

Panganiban, A. T., and Temin, H. M. (1984). The retrovirus pol gene encodes a product required for DNA integration: identification of a retrovirus int locus. *Proc Natl Acad Sci U S A* **81**(24), 7885-9.

Pantaleo, G., Graziosi, C., and Fauci, A. S. (1993). New concepts in the immunopathogenesis of human immunodeficiency virus infection. *N Engl J Med* **328**(5), 327-35.

Pantophlet, R., and Burton, D. R. (2006). GP120: target for neutralizing HIV-1 antibodies. *Annu Rev Immunol* **24**, 739-69.

Papanikolopoulou, K., Teixeira, S., Belrhali, H., Forsyth, V. T., Mitraki, A., and van Raaij, M. J. (2004). Adenovirus fibre shaft sequences fold into the native triple beta-spiral fold when N-terminally fused to the bacteriophage T4 fibritin foldon trimerisation motif. *J Mol Biol* **342**(1), 219-27.

Parada, C. A., and Roeder, R. G. (1999). A novel RNA polymerase II-containing complex potentiates Tat-enhanced HIV-1 transcription. *Embo J* **18**(13), 3688-701.

Parekh, B. S., Pau, C. P., Granade, T. C., Rayfield, M., De Cock, K. M., Gayle, H., Schochetman, G., and George, J. R. (1991). Oligomeric nature of transmembrane glycoproteins of HIV-2: procedures for their efficient dissociation and preparation of Western blots for diagnosis. *Aids* **5**(8), 1009-13.

Pascal, L. (1991). What happens when science goes bad. The corruption of science and the origin of AIDS: a study in spontaneous generation. *Wollongong: University of Wollongong Science and Technology Analysis Research Programme* **9**.

Paxton, W. A., Martin, S. R., Tse, D., O'Brien, T. R., Skurnick, J., VanDevanter, N. L., Padian, N., Braun, J. F., Kotler, D. P., Wolinsky, S. M., and Koup, R. A. (1996). Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. *Nat Med* **2**(4), 412-7.

Pearson, W. R., Robins, G., and Zhang, T. (1999). Generalized neighbor-joining: more reliable phylogenetic tree reconstruction. *Mol Biol Evol* **16**(6), 806-16.

Peeters, M., Courgnaud, V., Abela, B., Auzel, P., Pourrut, X., Bibollet-Ruche, F., Loul, S., Liegeois, F., Butel, C., Koulagna, D., Mpoudi-Ngole, E., Shaw, G. M., Hahn, B. H., and Delaporte, E. (2002). Risk to human health from a plethora of simian immunodeficiency viruses in primate bushmeat. *Emerg Infect Dis* **8**(5), 451-7.

Peeters, M., Honore, C., Huet, T., Bedjabaga, L., Ossari, S., Bussi, P., Cooper, R. W., and Delaporte, E. (1989). Isolation and partial characterization of an HIV-related virus occurring naturally in chimpanzees in Gabon. *AIDS* **3**(10), 625-30.

Peeters, M., Koumare, B., Mulanga, C., Brengues, C., Mounirou, B., Bougoudogo, F., Ravel, S., Bibollet-Ruche, F., and Delaporte, E. (1998). Genetic subtypes of HIV type 1 and HIV type 2 strains in commercial sex workers from Bamako, Mali. *AIDS Res Hum Retroviruses* **14**(1), 51-8.

Peeters, M., Toure-Kane, C., and Nkengasong, J. N. (2003). Genetic diversity of HIV in Africa: impact on diagnosis, treatment, vaccine development and trials. *AIDS* **17**(18), 2547-60.

Pepin, J., Dunn, D., Gaye, I., Alonso, P., Egboga, A., Tedder, R., Piot, P., Berry, N., Schellenberg, D., Whittle, H., and et al. (1991). HIV-2 infection among prostitutes working in The Gambia: association with serological evidence of genital ulcer diseases and with generalized lymphadenopathy. *AIDS* **5**(1), 69-75.

Pepin, J., Quigley, M., Todd, J., Gaye, I., Janneh, M., Van Dyck, E., Piot, P., and Whittle, H. (1992). Association between HIV-2 infection and genital ulcer diseases among male sexually transmitted disease patients in The Gambia. *AIDS* **6**(5), 489-93.

Perach, M., Rubinek, T., and Hizi, A. (1995). Resistance to nucleoside analogs of selective mutants of human immunodeficiency virus type 2 reverse transcriptase. *J Virol* **69**(1), 509-12.

Perotti, M. E., Tan, X., and Phillips, D. M. (1996). Directional budding of human immunodeficiency virus from monocytes. *J Virol* **70**(9), 5916-21.

Peruzzi, F. (2006). The multiple functions of HIV-1 Tat: proliferation versus apoptosis. *Front Biosci* **11**, 708-17.

Peterson, A., and Seed, B. (1988). Genetic analysis of monoclonal antibody and HIV binding sites on the human lymphocyte antigen CD4. *Cell* **54**(1), 65-72.

Pfutzner, A., Dietrich, U., von Eichel, U., von Briesen, H., Brede, H. D., Maniar, J. K., and Rubsamen-Waigmann, H. (1992). HIV-1 and HIV-2 infections in a high-risk population in Bombay, India: evidence for the spread of HIV-2 and presence of a divergent HIV-1 subtype. *J Acquir Immune Defic Syndr* **5**(10), 972-7.

Pichova, I., Weber, J., Litera, J., Konvalinka, J., Vondrasek, J., Soucek, M., Strop, P., Majer, P., Heuser, A. M., and Kraeusslich, H. G. (1997). Peptide inhibitors of HIV-1 and HIV-2 proteases: a comparative study. *Leukemia* **11 Suppl 3**, 120-2.

Pieniazek, D., Ellenberger, D., Janini, L. M., Ramos, A. C., Nkengasong, J., Sassan-Morokro, M., Hu, D. J., Coulibally, I. M., Ekpini, E., Bandea, C., Tanuri, A., Greenberg, A. E., Wiktor, S. Z., and Rayfield, M. A. (1999). Predominance of human immunodeficiency virus type 2 subtype B in Abidjan, Ivory Coast. *AIDS Res Hum Retroviruses* **15**(6), 603-8.

Piguet, V., and Trono, D. (1999). The Nef protein of primate lentiviruses. *Rev Med Virol* **9**(2), 111-20.

Pilgrim, A. K., Pantaleo, G., Cohen, O. J., Fink, L. M., Zhou, J. Y., Zhou, J. T., Bolognesi, D. P., Fauci, A. S., and Montefiori, D. C. (1997). Neutralizing antibody responses to human immunodeficiency virus type 1 in primary infection and long-term-nonprogressive infection. *J Infect Dis* **176**(4), 924-32.

Pinter, A., Honnen, W. J., He, Y., Gorny, M. K., Zolla-Pazner, S., and Kayman, S. C. (2004). The V1/V2 domain of gp120 is a global regulator of the sensitivity of primary human immunodeficiency virus type 1 isolates to neutralization by antibodies commonly induced upon infection. *J Virol* **78**(10), 5205-15.

Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B., and Kabat, D. (1998). Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J Virol* **72**(4), 2855-64.

Pleskoff, O., Treboute, C., and Alizon, M. (1998). The cytomegalovirus-encoded chemokine receptor US28 can enhance cell-cell fusion mediated by different viral proteins. *J Virol* **72**(8), 6389-97.

Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D., and Gallo, R. C. (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci U S A* **77**(12), 7415-9.

Poignard, P., Fouts, T., Naniche, D., Moore, J. P., and Sattentau, Q. J. (1996). Neutralizing antibodies to human immunodeficiency virus type-1 gp120 induce envelope glycoprotein subunit dissociation. *J Exp Med* **183**(2), 473-84.

Pollakis, G., Kang, S., Kliphuis, A., Chalaby, M. I., Goudsmit, J., and Paxton, W. A. (2001). N-linked glycosylation of the HIV type-1 gp120 envelope glycoprotein as a major determinant of CCR5 and CXCR4 coreceptor utilization. *J Biol Chem* **276**(16), 13433-41.

Popik, W., and Alce, T. M. (2004). CD4 receptor localized to non-raft membrane microdomains supports HIV-1 entry. Identification of a novel raft localization marker in CD4. *J Biol Chem* **279**(1), 704-12.

Popper, S. D. S., A. Travers, K. Gueye-Ndiaye, A. Mboup, S. Essex, M. Kanki, P (1999). Lower Human Immunodeficiency Virus (HIV) Type 2 Viral Load Reflects the Difference in Pathogenicity of HIV-1 and HIV-2. *The Journal of Infectious Diseases* **180**, 1116-21.

Popper, S. J., Sarr, A. D., Gueye-Ndiaye, A., Mboup, S., Essex, M. E., and Kanki, P. J. (2000). Low plasma human immunodeficiency virus type 2 viral load is independent of proviral load: low virus production in vivo. *J Virol* **74**(3), 1554-7.

Potter, S. J., Chew, C. B., Steain, M., Dwyer, D. E., and Saksena, N. K. (2004). Obstacles to successful antiretroviral treatment of HIV-1 infection: problems & perspectives. *Indian J Med Res* **119**(6), 217-37.

Poulsen, A. G., Aaby, P., Gottschau, A., Kvinesdal, B. B., Dias, F., Molbak, K., and Lauritzen, E. (1993). HIV-2 infection in Bissau, West Africa, 1987-1989: incidence, prevalences, and routes of transmission. *J Acquir Immune Defic Syndr* **6**(8), 941-8.

Poulsen, A. G., Aaby, P., Jensen, H., and Dias, F. (2000). Risk factors for HIV-2 seropositivity among older people in Guinea-Bissau. A search for the early history of HIV-2 infection. *Scand J Infect Dis* **32**(2), 169-75.

Poulsen, A. G., Aaby, P., Larsen, O., Jensen, H., Naucler, A., Lisse, I. M., Christiansen, C. B., Dias, F., and Melbye, M. (1997). 9-year HIV-2-associated mortality in an urban community in Bissau, west Africa. *Lancet* **349**(9056), 911-4.

Poulsen, A. G., Kvinesdal, B., Aaby, P., Molbak, K., Frederiksen, K., Dias, F., and Lauritzen, E. (1989). Prevalence of and mortality from human immunodeficiency virus type 2 in Bissau, West Africa. *Lancet* **1**(8642), 827-31.

Price, D. A., Goulder, P. J., Klenerman, P., Sewell, A. K., Easterbrook, P. J., Troop, M., Bangham, C. R., and Phillips, R. E. (1997). Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc Natl Acad Sci U S A* **94**(5), 1890-5.

Pugliese, A., Vidotto, V., Beltramo, T., Petrini, S., and Torre, D. (2005). A review of HIV-1 Tat protein biological effects. *Cell Biochem Funct* **23**(4), 223-7.

Qiao, Z. S., Kim, M., Reinhold, B., Montefiori, D., Wang, J. H., and Reinherz, E. L. (2005). Design, expression, and immunogenicity of a soluble HIV trimeric envelope fragment adopting a prefusion gp41 configuration. *J Biol Chem* **280**(24), 23138-46.

Quinn, T. C., Glasser, D., Cannon, R. O., Matuszak, D. L., Dunning, R. W., Kline, R. L., Campbell, C. H., Israel, E., Fauci, A. S., and Hook, E. W., 3rd (1988). Human immunodeficiency virus infection among patients attending clinics for sexually transmitted diseases. *N Engl J Med* **318**(4), 197-203.

Rafalski, M., Lear, J. D., and DeGrado, W. F. (1990). Phospholipid interactions of synthetic peptides representing the N-terminus of HIV gp41. *Biochemistry* **29**(34), 7917-22.

Rambaut, A., Robertson, D. L., Pybus, O. G., Peeters, M., and Holmes, E. C. (2001). Human immunodeficiency virus. Phylogeny and the origin of HIV-1. *Nature* **410**(6832), 1047-8.

Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., and et al. (1985). Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* **313**(6000), 277-84.

Raviv, Y., Viard, M., Bess, J., Jr., and Blumenthal, R. (2002). Quantitative measurement of fusion of HIV-1 and SIV with cultured cells using photosensitized labeling. *Virology* **293**(2), 243-51.

Reeves, J. D., Gallo, S. A., Ahmad, N., Miamidian, J. L., Harvey, P. E., Sharron, M., Pohlmann, S., Sfakianos, J. N., Derdeyn, C. A., Blumenthal, R., Hunter, E., and Doms, R. W. (2002). Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. *Proc Natl Acad Sci U S A* **99**(25), 16249-54.

Reeves, J. D., Hibbitts, S., Simmons, G., McKnight, A., Azevedo-Pereira, J. M., Moniz-Pereira, J., and Clapham, P. R. (1999). Primary human immunodeficiency virus type 2 (HIV-2) isolates infect CD4-negative cells via CCR5 and CXCR4: comparison with HIV-1 and simian immunodeficiency virus and relevance to cell tropism in vivo. *J Virol* **73**(9), 7795-804.

Reeves, J. D., McKnight, A., Potempa, S., Simmons, G., Gray, P. W., Power, C. A., Wells, T., Weiss, R. A., and Talbot, S. J. (1997). CD4-independent infection by HIV-2 (ROD/B): use of the 7-transmembrane receptors CXCR-4, CCR-3, and V28 for entry. *Virology* **231**(1), 130-4.

Reeves, J. D., and Piefer, A. J. (2005). Emerging drug targets for antiretroviral therapy. *Drugs* **65**(13), 1747-66.

Rey, M., Krust, B., Laurent, A. G., Montagnier, L., and Hovanessian, A. G. (1989). Characterisation of Human Immunodeficiency Virus Type 2 Envelope Glycoproteins: Dimerisation of the Glycoprotein Precursor during Processing. *Journal of Virology* **63**(2), 647-658.

Rey, M., Laurent, A. G., McClure, J., Krust, B., Montagnier, L., and Hovanessian, A. G. (1990). Transmembrane Envelope Glycoproteins of Human Immunodeficiency Virus Type 2 and Simian Immunodeficiency Virus SIV-mac Exist as Homodimers. *Journal of Virology* **64**(2), 922-926.

Reynard, F., Willkomm, N., Fatmi, A., Vallon-Eberhard, A., Verrier, B., and Bedin, F. (2006). Characterization of the antibody response elicited by HIV-1 Env glycomutants in rabbits. *Vaccine*.

Ricard, D., Wilkins, A., N'Gum, P. T., Hayes, R., Morgan, G., Da Silva, A. P., and Whittle, H. (1994). The effects of HIV-2 infection in a rural area of Guinea-Bissau. *AIDS* **8**(7), 977-82.

Richman, D. D., and Bozzette, S. A. (1994). The impact of the syncytium-inducing phenotype of human immunodeficiency virus on disease progression. *J Infect Dis* **169**(5), 968-74.

Rimsky, L. T., Shugars, D. C., and Matthews, T. J. (1998). Determinants of human immunodeficiency virus type 1 resistance to gp41-derived inhibitory peptides. *J Virol* **72**(2), 986-93.

Rizzuto, C., and Sodroski, J. (2000). Fine definition of a conserved CCR5-binding region on the human immunodeficiency virus type 1 glycoprotein 120. *AIDS Res Hum Retroviruses* **16**(8), 741-9.

Rizzuto, C. D., Wyatt, R., Hernandez-Ramos, N., Sun, Y., Kwong, P. D., Hendrickson, W. A., and Sodroski, J. (1998). A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* **280**(5371), 1949-53.

Robertson, D. L., Anderson, J. P., Bradac, J. A., Carr, J. K., Foley, B., Funkhouser, R. K., Gao, F., Hahn, B. H., Kalish, M. L., Kuiken, C., Learn, G. H., Leitner, T., McCutchan, F., Osmanov, S., Peeters, M., Pieniazek, D., Salminen, M., Sharp, P. M., Wolinsky, S., and Korber, B. (2000). HIV-1 nomenclature proposal. *Science* **288**(5463), 55-6.

Robey, W. G., Safai, B., Oroszlan, S., Arthur, L. O., Gonda, M. A., Gallo, R. C., and Fischinger, P. J. (1985). Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients. *Science* **228**(4699), 593-5.

Robinson, H. L., Montefiori, D. C., Johnson, R. P., Manson, K. H., Kalish, M. L., Lifson, J. D., Rizvi, T. A., Lu, S., Hu, S. L., Mazzara, G. P., Panicali, D. L., Herndon, J. G., Glickman, R., Candido, M. A., Lydy, S. L., Wyand, M. S., and McClure, H. M. (1999). Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat Med* **5**(5), 526-34.

Robinson, J., and Ho, D. (Unpublished data).

Rodes, B., Toro, C., Jimenez, V., and Soriano, V. (2005). Viral response to antiretroviral therapy in a patient coinfected with HIV type 1 and type 2. *Clin Infect Dis* **41**(2), e19-21.

Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A., and Walker, B. D. (1997). Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* **278**(5342), 1447-50.

Rosser, M. P., Xia, W., Hartsell, S., McCaman, M., Zhu, Y., Wang, S., Harvey, S., Bringmann, P., and Cobb, R. R. (2005). Transient transfection of CHO-K1-S using serum-free medium in suspension: a rapid mammalian protein expression system. *Protein Expr Purif* **40**(2), 237-43.

Rost, B., Fariselli, P., and Casadio, R. (1996). Topology prediction for helical transmembrane proteins at 86% accuracy. *Protein Sci* **5**(8), 1704-18.

Rost, B., and Sander, C. (1993). Prediction of protein secondary structure at better than 70% accuracy. *J Mol Biol* **232**(2), 584-99.

Rowland-Jones, S., Sutton, J., Ariyoshi, K., Dong, T., Gotch, F., McAdam, S., Whitby, D., Sabally, S., Gallimore, A., Corrah, T., and et al. (1995). HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. *Nat Med* **1**(1), 59-64.

Rubsamen-Waigmann, H., Briesen, H. V., Maniar, J. K., Rao, P. K., Scholz, C., and Pfutzner, A. (1991). Spread of HIV-2 in India. *Lancet* **337**(8740), 550-1.

Rucker, J., Edinger, A. L., Sharron, M., Samson, M., Lee, B., Berson, J. F., Yi, Y., Margulies, B., Collman, R. G., Doranz, B. J., Parmentier, M., and Doms, R. W. (1997). Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. *J Virol* **71**(12), 8999-9007.

Russell, R. J., Gamblin, S. J., Haire, L. F., Stevens, D. J., Xiao, B., Ha, Y., and Skehel, J. J. (2004). H1 and H7 influenza haemagglutinin structures extend a structural classification of haemagglutinin subtypes. *Virology* **325**(2), 287-96.

Russell, R. J., Haire, L. F., Stevens, D. J., Collins, P. J., Lin, Y. P., Blackburn, G. M., Hay, A. J., Gamblin, S. J., and Skehel, J. J. (2006). The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. *Nature* **443**(7107), 45-9.

Ryser, H. J., and Fluckiger, R. (2005). Progress in targeting HIV-1 entry. *Drug Discov Today* **10**(16), 1085-94.

Ryu, S. E., Kwong, P. D., Truneh, A., Porter, T. G., Arthos, J., Rosenberg, M., Dai, X. P., Xuong, N. H., Axel, R., Sweet, R. W., and et al. (1990). Crystal structure of an HIV-binding recombinant fragment of human CD4. *Nature* **348**(6300), 419-26.

Sackett, K., and Shai, Y. (2005). The HIV fusion peptide adopts intermolecular parallel beta-sheet structure in membranes when stabilized by the adjacent N-terminal heptad repeat: a 13C FTIR study. *J Mol Biol* **350**(4), 790-805.

Safrin, S., and Grunfeld, C. (1999). Fat distribution and metabolic changes in patients with HIV infection. *AIDS* **13**(18), 2493-505.

Saha, K., Zhang, J., Gupta, A., Dave, R., Yimen, M., and Zerhouni, B. (2001). Isolation of primary HIV-1 that target CD8+ T lymphocytes using CD8 as a receptor. *Nat Med* **7**(1), 65-72.

Sakai, H., Kawamura, M., Sakuragi, J., Sakuragi, S., Shibata, R., Ishimoto, A., Ono, N., Ueda, S., and Adachi, A. (1993). Integration is essential for efficient gene expression of human immunodeficiency virus type 1. *J Virol* **67**(3), 1169-74.

Saksena, N. K., and Haddad, D. N. (2003). Viral reservoirs an impediment to HAART: new strategies to eliminate HIV-1. *Curr Drug Targets Infect Disord* **3**(2), 179-206.

Salmon-Ceron, D., Excler, J. L., Sicard, D., Blanche, P., Finkielstzjen, L., Gluckman, J. C., Autran, B., Matthews, T. J., Meignier, B., Kieny, M. P., and et al. (1995). Safety and immunogenicity of a recombinant HIV type 1 glycoprotein 160 boosted by a V3 synthetic peptide in HIV-negative volunteers. *AIDS Res Hum Retroviruses* **11**(12), 1479-86.

Salzwedel, K., West, J. T., and Hunter, E. (1999). A conserved tryptophan-rich motif in the membrane-proximal region of the human immunodeficiency virus type 1 gp41 ectodomain is important for Env-mediated fusion and virus infectivity. *J Virol* **73**(3), 2469-80.

Sambrook, J., and Russell, D. W. (2001). "Molecular cloning : a laboratory manual." 3rd Edition ed. Cold Spring Harbor Laboratory Press, 2001, New York.

Samson, M., Edinger, A. L., Stordeur, P., Rucker, J., Verhasselt, V., Sharron, M., Govaerts, C., Mollereau, C., Vassart, G., Doms, R. W., and Parmentier, M. (1998). ChemR23, a putative chemoattractant receptor, is expressed in monocyte-derived dendritic cells and macrophages and is a coreceptor for SIV and some primary HIV-1 strains. *Eur J Immunol* **28**(5), 1689-700.

Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., Muyldermans, G., Verhofstede, C., Burtonboy, G., Georges, M., Imai, T., Rana, S., Yi, Y., Smyth, R. J., Collman, R. G., Doms, R. W., Vassart, G., and Parmentier, M. (1996). Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**(6593), 722-5.

Sanders, R. W., Venturi, M., Schiffner, L., Kalyanaraman, R., Katinger, H., Lloyd, K. O., Kwong, P. D., and Moore, J. P. (2002). The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. *J Virol* **76**(14), 7293-305.

Sankale, J. L., De La Tour, R. S., Marlink, R. G., Scheib, R., Mboup, S., Essex, M. E., and Kanki, P. J. (1996). Distinct quasi-species in the blood and the brain of an HIV-2-infected individual. *Virology* **226**(2), 418-23.

Sankale, J. L., Mboup, S., Essex, M. E., and Kanki, P. J. (1998). Genetic characterization of viral quasispecies in blood and cervical secretions of HIV-1- and HIV-2-infected women. *AIDS Res Hum Retroviruses* **14**(16), 1473-81.

Santiago, M. L., Range, F., Keele, B. F., Li, Y., Bailes, E., Bibollet-Ruche, F., Fruteau, C., Noe, R., Peeters, M., Brookfield, J. F., Shaw, G. M., Sharp, P. M., and Hahn, B. H. (2005). Simian Immunodeficiency Virus Infection in Free-Ranging Sooty Mangabeys (Cercocebus atys atys) from the Tai Forest, Cote d'Ivoire: Implications for the Origin of Epidemic Human Immunodeficiency Virus Type 2. *J Virol* **79**(19), 12515-27.

Santos-Ferreira, M. O., Cohen, T., Lourenco, M. H., Almeida, M. J., Chamaret, S., and Montagnier, L. (1990). A study of seroprevalence of HIV-1 and HIV-2 in six provinces of People's Republic of Angola: clues to the spread of HIV infection. *J Acquir Immune Defic Syndr* **3**(8), 780-6.

Sarr, A. D., Popper, S., Thior, I., Hamel, D. J., Sankale, J. L., Siby, T., Marlink, R., Essex, M., Mboup, S., and Kanki, P. (1999). Relation between HIV-2 proviral load and CD4+ lymphocyte count differs in monotypic and dual HIV infections. *J Hum Virol* **2**(1), 45-51.

Sarr, A. D., Sankale, J. L., Gueye-Ndiaye, A., Essex, M., Mboup, S., and Kanki, P. J. (2000). Genetic analysis of HIV type 2 in monotypic and dual HIV infections. *AIDS Res Hum Retroviruses* **16**(3), 295-8.

Sassan-Morokro, M., Greenberg, A. E., Coulibaly, I. M., Coulibaly, D., Sidibe, K., Ackah, A., Tossou, O., Gnaore, E., Wiktor, S. Z., and De Cock, K. M. (1996). High rates of sexual contact with female sex workers, sexually transmitted diseases, and condom neglect among HIV-infected and uninfected men with tuberculosis in Abidjan, Cote d'Ivoire. *J Acquir Immune Defic Syndr Hum Retrovirol* **11**(2), 183-7.

Sattentau, Q. J. (1992). CD4 activation of HIV fusion. *Int J Cell Cloning* **10**(6), 323-32.

Sattentau, Q. J., and Moore, J. P. (1991). Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. *J Exp Med* **174**(2), 407-15.

Sattentau, Q. J., Moore, J. P., Vignaux, F., Traincard, F., and Poignard, P. (1993). Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. *J Virol* **67**(12), 7383-93.

Sattentau, Q. J., Zolla-Pazner, S., and Poignard, P. (1995). Epitope exposure on functional, oligomeric HIV-1 gp41 molecules. *Virology* **206**(1), 713-7.

Sawai, E. T., Baur, A., Struble, H., Peterlin, B. M., Levy, J. A., and Cheng-Mayer, C. (1994). Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. *Proc Natl Acad Sci U S A* **91**(4), 1539-43.

Scanlan, C. N., Pantophlet, R., Wormald, M. R., Ollmann Saphire, E., Stanfield, R., Wilson, I. A., Katinger, H., Dwek, R. A., Rudd, P. M., and Burton, D. R. (2002). The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of alpha1-->2 mannose residues on the outer face of gp120. *J Virol* **76**(14), 7306-21.

Schaal, H., Klein, M., Gehrmann, P., Adams, O., and Scheid, A. (1995). Requirement of N-terminal amino acid residues of gp41 for human immunodeficiency virus type 1-mediated cell fusion. *J Virol* **69**(6), 3308-14.

Schacker, T., Collier, A. C., Hughes, J., Shea, T., and Corey, L. (1996). Clinical and epidemiologic features of primary HIV infection. *Ann Intern Med* **125**(4), 257-64.

Schacker, T. W., Hughes, J. P., Shea, T., Coombs, R. W., and Corey, L. (1998). Biological and virologic characteristics of primary HIV infection. *Ann Intern Med* **128**(8), 613-20.

Schim van der Loeff, M. F., Jaffar, S., Aveika, A. A., Sabally, S., Corrah, T., Harding, E., Alabi, A., Bayang, A., Ariyoshi, K., and Whittle, H. C. (2002). Mortality of HIV-1, HIV-2 and HIV-1/HIV-2 dually infected patients in a clinic-based cohort in The Gambia. *Aids* **16**(13), 1775-83.

Schindler, M., Wurfl, S., Benaroch, P., Greenough, T. C., Daniels, R., Easterbrook, P., Brenner, M., Munch, J., and Kirchhoff, F. (2003). Down-modulation of mature major histocompatibility complex class II and up-regulation of invariant chain cell surface expression are well-conserved functions of human and simian immunodeficiency virus nef alleles. *J Virol* **77**(19), 10548-56.

Schols, D., and De Clercq, E. (1996). Human immunodeficiency virus type 1 gp120 induces anergy in human peripheral blood lymphocytes by inducing interleukin-10 production. *J Virol* **70**(8), 4953-60.

Schols, D., and De Clercq, E. (1998). The simian immunodeficiency virus mnd(GB-1) strain uses CXCR4, not CCR5, as coreceptor for entry in human cells. *J Gen Virol* **79 (Pt 9)**, 2203-5.

Schonning, K., Jansson, B., Olofsson, S., and Hansen, J. E. (1996). Rapid selection for an N-linked oligosaccharide by monoclonal antibodies directed against the V3 loop of human immunodeficiency virus type 1. *J Gen Virol* **77 (Pt 4)**, 753-8.

Schooley, R. T., Merigan, T. C., Gaut, P., Hirsch, M. S., Holodniy, M., Flynn, T., Liu, S., Byington, R. E., Henochowicz, S., Gubish, E., and et al. (1990). Recombinant soluble CD4 therapy in patients with the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. A phase I-II escalating dosage trial. *Ann Intern Med* **112**(4), 247-53.

Schuck, P. (2000). Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. *Biophys J* **78**(3), 1606-19.

Schulke, N., Vesanen, M. S., Sanders, R. W., Zhu, P., Lu, M., Anselma, D. J., Villa, A. R., Parren, P. W., Binley, J. M., Roux, K. H., Maddon, P. J., Moore, J. P., and Olson, W. C. (2002). Oligomeric and conformational properties of a proteolytically mature, disulfide-stabilized human immunodeficiency virus type 1 gp140 envelope glycoprotein. *J Virol* **76**(15), 7760-76.

Schulz, T. F., Jameson, B. A., Lopalco, L., Siccardi, A. G., Weiss, R. A., and Moore, J. P. (1992). Conserved structural features in the interaction between retroviral surface and transmembrane glycoproteins? *AIDS Res Hum Retroviruses* **8**(9), 1571-80.

Schutten, M., van der Ende, M. E., and Osterhaus, A. D. (2000). Antiretroviral therapy in patients with dual infection with human immunodeficiency virus types 1 and 2. *N Engl J Med* **342**(23), 1758-60.

Scott-Algara, D., Truong, L. X., Versmisse, P., David, A., Luong, T. T., Nguyen, N. V., Theodorou, I., Barre-Sinoussi, F., and Pancino, G. (2003). Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. *J Immunol* **171**(11), 5663-7.

Sekigawa, I., Kaneko, H., Pin Neoh, L., Takeda-Hirokawa, N., Akimoto, H., Hishikawa, T., Hashimoto, H., Hirose, S., Yamamoto, N., and Kaneko, Y. (1998). Differences of HIV Envelope Protein between HIV-1 and HIV-2: Possible Relation to the Lower Virulence of HIV-2. *Viral Immunology* **11**(1), 1-8.

Shanmugam, V., Switzer, W. M., Nkengasong, J. N., Garcia-Lerma, G., Green, T. A., Ekpini, E., Sassan-Morokro, M., Antunes, F., Manshino, K., Soriano, V., Wiktor, S. Z., and Heneine, W. (2000). Lower HIV-2 plasma viral loads may explain differences between the natural histories of HIV-1 and HIV-2 infections. *J Acquir Immune Defic Syndr* **24**(3), 257-63.

Sharp, P. M., Bailes, E., Chaudhuri, R. R., Rodenburg, C. M., Santiago, M. O., and Hahn, B. H. (2001). The origins of acquired immune deficiency syndrome viruses: where and when? *Philos Trans R Soc Lond B Biol Sci* **356**(1410), 867-76.

Sharp, P. M., Bailes, E., Stevenson, M., Emerman, M., and Hahn, B. H. (1996). Gene acquisition in HIV and SIV. *Nature* **383**(6601), 586-7.

Sharp, P. M., Robertson, D. L., and Hahn, B. H. (1995). Cross-species transmission and recombination of 'AIDS' viruses. *Philos Trans R Soc Lond B Biol Sci* **349**(1327), 41-7.

Sharp, P. M., Shaw, G. M., and Hahn, B. H. (2005). Simian immunodeficiency virus infection of chimpanzees. *J Virol* **79**(7), 3891-902.

Shea, A., Sarr, D. A., Jones, N., Penning, L., Eisen, G., Gueye-Ndiaye, A., Mboup, S., Kanki, P., and Cao, H. (2004). CCR5 receptor expression is down-regulated in HIV type 2 infection: implication for viral control and protection. *AIDS Res Hum Retroviruses* **20**(6), 630-5.

Shearer, W. T., Israel, R. J., Starr, S., Fletcher, C. V., Wara, D., Rathore, M., Church, J., DeVille, J., Fenton, T., Graham, B., Samson, P., Staprans, S., McNamara, J., Moye, J., Maddon, P. J., and Olson, W. C. (2000). Recombinant CD4-IgG2 in human immunodeficiency virus type 1-infected children: phase 1/2 study. The Pediatric AIDS Clinical Trials Group Protocol 351 Study Team. *J Infect Dis* **182**(6), 1774-9.

Sheehy, A. M., Gaddis, N. C., Choi, J. D., and Malim, M. H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418**(6898), 646-50.

Sheehy, A. M., Gaddis, N. C., and Malim, M. H. (2003). The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nat Med* **9**(11), 1404-7.

Shehu-Xhilaga, M., Hill, M., Marshall, J. A., Kappes, J., Crowe, S. M., and Mak, J. (2002). The conformation of the mature dimeric human immunodeficiency virus type 1 RNA genome requires packaging of pol protein. *J Virol* **76**(9), 4331-40.

Shibata, R., Igarashi, T., Haigwood, N., Buckler-White, A., Ogert, R., Ross, W., Willey, R., Cho, M. W., and Martin, M. A. (1999). Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nat Med* **5**(2), 204-10.

Shibuya, N., Goldstein, I. J., Van Damme, E. J., and Peumans, W. J. (1988). Binding properties of a mannose-specific lectin from the snowdrop (Galanthus nivalis) bulb. *J Biol Chem* **263**(2), 728-34.

Shilatifard, A., Merkle, R. K., Helland, D. E., Welles, J. L., Haseltine, W. A., and Cummings, R. D. (1993). Complex-type N-linked oligosaccharides of gp120 from human immunodeficiency virus type 1 contain sulfated N-acetylglucosamine. *J Virol* **67**(2), 943-52.

Shimizu, N., Soda, Y., Kanbe, K., Liu, H. Y., Jinno, A., Kitamura, T., and Hoshino, H. (1999). An orphan G protein-coupled receptor, GPR1, acts as a coreceptor to allow replication of human immunodeficiency virus types 1 and 2 in brain-derived cells. *J Virol* **73**(6), 5231-9.

Shimizu, N., Soda, Y., Kanbe, K., Liu, H. Y., Mukai, R., Kitamura, T., and Hoshino, H. (2000). A putative G protein-coupled receptor, RDC1, is a novel coreceptor for human and simian immunodeficiency viruses. *J Virol* **74**(2), 619-26.

Shinoda, K., Xin, K. Q., Kojima, Y., Saha, S., and Okuda, K. (2006). Robust HIV-specific immune responses were induced by DNA vaccine prime followed by attenuated recombinant vaccinia virus (LC16m8 strain) boost. *Clin Immunol* **119**(1), 32-7.

Shu, L. L., Lin, Y. P., Wright, S. M., Shortridge, K. F., and Webster, R. G. (1994). Evidence for interspecies transmission and reassortment of influenza A viruses in pigs in southern China. *Virology* **202**(2), 825-33.

Si, Z., Madani, N., Cox, J. M., Chruma, J. J., Klein, J. C., Schon, A., Phan, N., Wang, L., Biorn, A. C., Cocklin, S., Chaiken, I., Freire, E., Smith, A. B., 3rd, and Sodroski, J. G. (2004). Small-molecule inhibitors of HIV-1 entry block receptor-induced conformational changes in the viral envelope glycoproteins. *Proc Natl Acad Sci U S A* **101**(14), 5036-41.

Simmons, G., Reeves, J. D., Hibbitts, S., Stine, J. T., Gray, P. W., Proudfoot, A. E., and Clapham, P. R. (2000). Co-receptor use by HIV and inhibition of HIV infection by chemokine receptor ligands. *Immunol Rev* **177**, 112-26.

Simmons, G., Wilkinson, D., Reeves, J. D., Dittmar, M. T., Beddows, S., Weber, J., Carnegie, G., Desselberger, U., Gray, P. W., Weiss, R. A., and Clapham, P. R. (1996). Primary, syncytium-inducing human immunodeficiency virus type 1 isolates are dual-tropic and most can use either Lestr or CCR5 as coreceptors for virus entry. *J Virol* **70**(12), 8355-60.

Simon, F., Mauclere, P., Roques, P., Loussert-Ajaka, I., Muller-Trutwin, M. C., Saragosti, S., Georges-Courbot, M. C., Barre-Sinoussi, F., and Brun-Vezinet, F. (1998). Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nat Med* **4**(9), 1032-7.

Simon, J. H., and Malim, M. H. (1996). The human immunodeficiency virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes. *J Virol* **70**(8), 5297-305.

Sirois, S., Sing, T., and Chou, K. C. (2005). HIV-1 gp120 V3 loop for structure-based drug design. *Curr Protein Pept Sci* **6**(5), 413-22.

Skehel, J. J., and Wiley, D. C. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* **69**, 531-69.

Slepushkin, V. A., Melikyan, G. B., Sidorova, M. S., Chumakov, V. M., Andreev, S. M., Manulyan, R. A., and Karamov, E. V. (1990). Interaction of human immunodeficiency virus (HIV-1) fusion peptides with artificial lipid membranes. *Biochem Biophys Res Commun* **172**(2), 952-7.

Smith, M. W., Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Lomb, D. A., Goedert, J. J., O'Brien, T. R., Jacobson, L. P., Kaslow, R., Buchbinder, S., Vittinghoff, E., Vlahov, D., Hoots, K., Hilgartner, M. W., and O'Brien, S. J. (1997). Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC), ALIVE Study. *Science* **277**(5328), 959-65.

Smith, N. A., Shaw, T., Berry, N., Vella, C., Okorafor, L., Taylor, D., Ainsworth, J., Choudhury, A., Daniels, R. S., El-Gadi, S., Fakoya, A., Moyle, G., Oxford, J., Tedder, R., O'Shea, S., de Ruiter, A., and Breuer, J. (2001). Antiretroviral therapy for HIV-2 infected patients. *J Infect* **42**(2), 126-33.

Smith, S. W., Overbeek, R., Woese, C. R., Gilbert, W., and Gillevet, P. M. (1994). The genetic data environment an expandable GUI for multiple sequence analysis. *Comput Appl Biosci* **10**(6), 671-5.

Smyth, R. J., Yi, Y., Singh, A., and Collman, R. G. (1998). Determinants of entry cofactor utilization and tropism in a dualtropic human immunodeficiency virus type 1 primary isolate. *J Virol* **72**(5), 4478-84.

Sodroski, J., Goh, W. C., Rosen, C., Dayton, A., Terwilliger, E., and Haseltine, W. (1986). A second post-transcriptional trans-activator gene required for HTLV-III replication. *Nature* **321**(6068), 412-7.

Sol, N., Ferchal, F., Braun, J., Pleskoff, O., Treboute, C., Ansart, I., and Alizon, M. (1997). Usage of the coreceptors CCR-5, CCR-3, and CXCR-4 by primary and cell line-adapted human immunodeficiency virus type 2. *J Virol* **71**(11), 8237-44.

Sollner, T. H. (2004). Intracellular and viral membrane fusion: a uniting mechanism. *Curr Opin Cell Biol* **16**(4), 429-35.

Soriano, V., and de Mendoza, C. (2002). Genetic mechanisms of resistance to NRTI and NNRTI. *HIV Clin Trials* **3**(3), 237-48.

Soriano, V., Gomes, P., Heneine, W., Holguin, A., Doruana, M., Antunes, R., Mansinho, K., Switzer, W. M., Araujo, C., Shanmugam, V., Lourenco, H., Gonzalez-Lahoz, J., and Antunes, F. (2000). Human immunodeficiency virus type 2 (HIV-2) in Portugal: clinical spectrum, circulating subtypes, virus isolation, and plasma viral load. *J Med Virol* **61**(1), 111-6.

Soumelis, V., Scott, I., Gheyas, F., Bouhour, D., Cozon, G., Cotte, L., Huang, L., Levy, J. A., and Liu, Y. J. (2001). Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients. *Blood* **98**(4), 906-12.

Sourial, S., Warnmark, A., Nilsson, C., Bjorling, E., Achour, A., and Harris, R. A. (2005). Cloning, expression, and purification of HIV-2 gp125: A target for HIV vaccination. *Mol Biotechnol* **30**(2), 155-62.

Sousa, A. E., Carneiro, J., Meier-Schellersheim, M., Grossman, Z., and Victorino, R. M. (2002). CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. *J Immunol* **169**(6), 3400-6.

Spies, C. P., and Compans, R. W. (1993). Alternate pathways of secretion of simian immunodeficiency virus envelope glycoproteins. *J Virol* **67**(11), 6535-41.

Spies, C. P., Ritter, G. D., Jr., Mulligan, M. J., and Compans, R. W. (1994). Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein alters the conformation of the external domain. *J Virol* **68**(2), 585-91.

Sreerama, N., and Woody, R. W. (2000). Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal Biochem* **287**(2), 252-60.

Srivastava, I. K., Stamatatos, L., Kan, E., Vajdy, M., Lian, Y., Hilt, S., Martin, L., Vita, C., Zhu, P., Roux, K. H., Vojtech, L., Montefiori, D. C., Donnelly, J. J., Ulmer, J. B., and Barnett, S. W. (2003). Purification, Characterization, and Immunogenicity of a Soluble Trimeric Envelope Protein Containing a Partial Deletion of the V2 Loop Derived from SF162, an R5-Tropic Human Immunodeficiency Virus Type 1 Isolate. *Journal of Virology* **77**(20), 11244-11259.

Srivastava, I. K., Stamatatos, L., Legg, H., Kan, E., Fong, A., Coates, S., Leung, L., Wininger, M., Donnelly, J. J., Ulmer, J. B., and Barnett, S. W. (2002). Purification and Characterisation of Oligomeric Envelope Glycoprotein from a primary R5 Subtype Human Immunodeficiency Virus. *Journal of Virology* **76**(6), 2835-2847.

Stamm, W. E., Handsfield, H. H., Rompalo, A. M., Ashley, R. L., Roberts, P. L., and Corey, L. (1988). The association between genital ulcer disease and acquisition of HIV infection in homosexual men. *Jama* **260**(10), 1429-33.

Stanley, P. (1989). Chinese hamster Ovary Cell Mutants with Multiple Glycosylation Defects for production of Glycoproteins with Minimal carbohydrate Heterogeneity. *Molecular and Cellular Biology* **9**(2), 377-383.

Staprans, S. I., Hamilton, B. L., Follansbee, S. E., Elbeik, T., Barbosa, P., Grant, R. M., and Feinberg, M. B. (1995). Activation of virus replication after vaccination of HIV-1-infected individuals. *J Exp Med* **182**(6), 1727-37.

Starcich, B. R., Hahn, B. H., Shaw, G. M., McNeely, P. D., Modrow, S., Wolf, H., Parks, E. S., Parks, W. P., Josephs, S. F., Gallo, R. C., and et al. (1986). Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. *Cell* **45**(5), 637-48.

Staropoli, I., Chanel, C., Girard, M., and Altmeyer, R. (2000). Processing, stability, and receptor binding properties of oligomeric envelope glycoprotein from a primary HIV-1 isolate. *J Biol Chem* **275**(45), 35137-45.

Strelkov, S. V., Tao, Y., Rossmann, M. G., Kurochkina, L. P., Shneider, M. M., and Mesyanzhinov, V. V. (1996). Preliminary crystallographic studies of bacteriophage T4 fibritin confirm a trimeric coiled-coil structure. *Virology* **219**(1), 190-4.

Strelkov, S. V., Tao, Y., Shneider, M. M., Mesyanzhinov, V. V., and Rossmann, M. G. (1998). Structure of bacteriophage T4 fibritin M: a troublesome packing arrangement. *Acta Crystallogr D Biol Crystallogr* **54 (Pt 5)**, 805-16.

Strizki, J. M., Xu, S., Wagner, N. E., Wojcik, L., Liu, J., Hou, Y., Endres, M., Palani, A., Shapiro, S., Clader, J. W., Greenlee, W. J., Tagat, J. R., McCombie, S., Cox, K., Fawzi, A. B., Chou, C. C., Pugliese-Sivo, C., Davies, L., Moreno, M. E., Ho, D. D., Trkola, A., Stoddart, C. A., Moore, J. P., Reyes, G. R., and Baroudy, B. M. (2001). SCH-C (SCH 351125), an orally bioavailable, small molecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection in vitro and in vivo. *Proc Natl Acad Sci U S A* **98**(22), 12718-23.

Suarez, T., Gallaher, W. R., Agirre, A., Goni, F. M., and Nieva, J. L. (2000). Membrane interface-interacting sequences within the ectodomain of the human immunodeficiency virus type 1 envelope glycoprotein: putative role during viral fusion. *J Virol* **74**(17), 8038-47.

Sullivan, M. T., Guido, E. A., Metler, R. P., Schable, C. A., Williams, A. E., and Stramer, S. L. (1998). Identification and characterization of an HIV-2 antibody-positive blood donor in the United States. *Transfusion* **38**(2), 189-93.

Swingler, S., Mann, A., Jacque, J., Brichacek, B., Sasseville, V. G., Williams, K., Lackner, A. A., Janoff, E. N., Wang, R., Fisher, D., and Stevenson, M. (1999). HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. *Nat Med* **5**(9), 997-103.

Switzer, W. M., Wiktor, S., Soriano, V., Silva-Graca, A., Mansinho, K., Coulibaly, I. M., Ekpini, E., Greenberg, A. E., Folks, T. M., and Heneine, W. (1998). Evidence of Nef truncation in human immunodeficiency virus type 2 infection. *J Infect Dis* **177**(1), 65-71.

Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., Kishimoto, T., and Nagasawa, T. (1998). The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* **393**(6685), 591-4.

Takeda, A., and Ennis, F. A. (1990). FcR-mediated enhancement of HIV-1 infection by antibody. *AIDS Res Hum Retroviruses* **6**(8), 999-1004.

Takeda, A., Robinson, J. E., Ho, D. D., Debouck, C., Haigwood, N. L., and Ennis, F. A. (1992). Distinction of human immunodeficiency virus type 1 neutralization and infection enhancement by human monoclonal antibodies to glycoprotein 120. *J Clin Invest* **89**(6), 1952-7.

Takehisa, J., Osei-Kwasi, M., Ayisi, N. K., Hishida, O., Miura, T., Igarashi, T., Brandful, J., Ampofo, W., Netty, V. B., Mensah, M., Yamashita, M., Ido, E., and Hayami, M. (1997). Phylogenetic analysis of HIV type 2 in Ghana and intrasubtype recombination in HIV type 2. *AIDS Res Hum Retroviruses* **13**(7), 621-3.

Tamamura, H., Xu, Y., Hattori, T., Zhang, X., Arakaki, R., Kanbara, K., Omagari, A., Otaka, A., Ibuka, T., Yamamoto, N., Nakashima, H., and Fujii, N. (1998). A low-molecular-weight inhibitor against the chemokine receptor CXCR4: a strong anti-HIV peptide T140. *Biochem Biophys Res Commun* **253**(3), 877-82.

Tamm, L. K. (2003). Hypothesis: spring-loaded boomerang mechanism of influenza hemagglutinin-mediated membrane fusion. *Biochim Biophys Acta* **1614**(1), 14-23.

Tan, K., Liu, J., Wang, J., Shen, S., and Lu, M. (1997). Atomic structure of a thermostable subdomain of HIV-1 gp41. *Proc Natl Acad Sci U S A* **94**(23), 12303-8.

Tao, Y., Strelkov, S. V., Mesyanzhinov, V. V., and Rossmann, M. G. (1997). Structure of bacteriophage T4 fibritin: a segmented coiled coil and the role of the C-terminal domain. *Structure* **5**(6), 789-98.

Tarrago-Litvak, L., Andreola, M. L., Nevinsky, G. A., Sarih-Cottin, L., and Litvak, S. (1994). The reverse transcriptase of HIV-1: from enzymology to therapeutic intervention. *Faseb J* **8**(8), 497-503.

Telzak, E. E., Chiasson, M. A., Bevier, P. J., Stoneburner, R. L., Castro, K. G., and Jaffe, H. W. (1993). HIV-1 seroconversion in patients with and without genital ulcer disease. A prospective study. *Ann Intern Med* **119**(12), 1181-6.

Temesgen, Z., and Wright, A. J. (1999). Antiretrovirals. *Mayo Clin Proc* **74**(12), 1284-301.

Tevi-Benissan, C., Okome, M., Makuwa, M., Nkoume, M. N., Lansoud-Soukate, J., Georges, A., Georges-Courbot, M. C., and Belec, L. (1998). HIV-2 infection and HIV-1/HIV-2 dual reactivity in patients with and without AIDS-related symptoms in Gabon. *Emerg Infect Dis* **4**(1), 130-1.

Thali, M., Moore, J. P., Furman, C., Charles, M., Ho, D. D., Robinson, J., and Sodroski, J. (1993). Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *J Virol* **67**(7), 3978-88.

Thomas, E. R., Shotton, C., Weiss, R. A., Clapham, P. R., and McKnight, A. (2003). CD4-dependent and CD4-independent HIV-2: consequences for neutralization. *Aids* **17**(3), 291-300.

Tisne, C. (2005). Structural bases of the annealing of primer tRNA(3Lys) to the HIV-1 viral RNA. *Curr HIV Res* **3**(2), 147-56.

Tomasselli, A. G., Hui, J. O., Sawyer, T. K., Staples, D. J., Bannow, C., Reardon, I. M., Howe, W. J., DeCamp, D. L., Craik, C. S., and Heinrikson, R. L. (1990). Specificity and inhibition of proteases from human immunodeficiency viruses 1 and 2. *J Biol Chem* **265**(24), 14675-83.

Tomiyama, H., Miwa, K., Shiga, H., Moore, Y. I., Oka, S., Iwamoto, A., Kaneko, Y., and Takiguchi, M. (1997). Evidence of presentation of multiple HIV-1 cytotoxic T lymphocyte epitopes by HLA-B*3501 molecules that are associated with the accelerated progression of AIDS. *J Immunol* **158**(10), 5026-34.

Toro, C., Soriano, V., Tuset, C., Aguilera, A., Caballero, E., de Lejarazu, R. O., and Rodes, B. (2002). Infection with retroviruses other than HIV-1 in Spain: a retrospective analysis for HIV-2, HTLV-I, and/or HTLV-II. *HIV Clin Trials* **3**(5), 397-402.

Travers, K., Mboup, S., Marlink, R., Gueye-Nidaye, A., Siby, T., Thior, I., Traore, I., Dieng-Sarr, A., Sankale, J. L., Mullins, C., and et al. (1995). Natural protection against HIV-1 infection provided by HIV-2. *Science* **268**(5217), 1612-5.

Tristem, M., Marshall, C., Karpas, A., Petrik, J., and Hill, F. (1990). Origin of vpx in lentiviruses. *Nature* **347**(6291), 341-2.

Tristem, M., Purvis, A., and Quicke, D. L. (1998). Complex evolutionary history of primate lentiviral vpr genes. *Virology* **240**(2), 232-7.

Trkola, A., Kuster, H., Rusert, P., Joos, B., Fischer, M., Leemann, C., Manrique, A., Huber, M., Rehr, M., Oxenius, A., Weber, R., Stiegler, G., Vcelar, B., Katinger, H., Aceto, L., and Gunthard, H. F. (2005). Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. *Nat Med* **11**(6), 615-22.

Trkola, A., Pomales, A. B., Yuan, H., Korber, B., Maddon, P. J., Allaway, G. P., Katinger, H., Barbas, C. F., 3rd, Burton, D. R., Ho, D. D., and et al. (1995). Cross-clade neutralization of primary isolates of human immunodeficiency virus type 1 by human monoclonal antibodies and tetrameric CD4-IgG. *J Virol* **69**(11), 6609-17.

Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J. P., and Katinger, H. (1996). Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J Virol* **70**(2), 1100-8.

Turner, B. G., and Summers, M. F. (1999). Structural biology of HIV. *J Mol Biol* **285**(1), 1-32.

Ulrich, R., Siakkou, H., and Kruger, D. H. (1992). Expression of HIV-2 Gag and Env antigens in E. coli. *Acta Virol* **36**(5), 491.

Valentine, F. T., Kundu, S., Haslett, P. A., Katzenstein, D., Beckett, L., Spino, C., Borucki, M., Vasquez, M., Smith, G., Korvick, J., Kagan, J., and Merigan, T. C. (1996). A randomized, placebo-controlled study of the immunogenicity of human immunodeficiency virus (HIV) rgp160 vaccine in HIV-infected subjects with > or = 400/mm3 CD4 T lymphocytes (AIDS Clinical Trials Group Protocol 137). *J Infect Dis* **173**(6), 1336-46.

Veazey, R. S., DeMaria, M., Chalifoux, L. V., Shvetz, D. E., Pauley, D. R., Knight, H. L., Rosenzweig, M., Johnson, R. P., Desrosiers, R. C., and Lackner, A. A. (1998). Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* **280**(5362), 427-31.

Veazey, R. S., Marx, P. A., and Lackner, A. A. (2003). Vaginal CD4+ T cells express high levels of CCR5 and are rapidly depleted in simian immunodeficiency virus infection. *J Infect Dis* **187**(5), 769-76.

Veronesi, R., Mazza, C. C., Santos Ferreira, M. O., and Lourenco, M. H. (1987). HIV-2 in Brazil. *Lancet* **2**(8555), 402.

Vidal, N., Peeters, M., Mulanga-Kabeya, C., Nzilambi, N., Robertson, D., Ilunga, W., Sema, H., Tshimanga, K., Bongo, B., and Delaporte, E. (2000). Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. *J Virol* **74**(22), 10498-507.

Vittinghoff, E., Douglas, J., Judson, F., McKirnan, D., MacQueen, K., and Buchbinder, S. P. (1999). Per-contact risk of human immunodeficiency virus transmission between male sexual partners. *Am J Epidemiol* **150**(3), 306-11.

Voltan, R., and Robert-Guroff, M. (2003). Live recombinant vectors for AIDS vaccine development. *Curr Mol Med* **3**(3), 273-84.

von Schwedler, U., Song, J., Aiken, C., and Trono, D. (1993). Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. *J Virol* **67**(8), 4945-55.

Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S., and Alizon, M. (1985). Nucleotide sequence of the AIDS virus, LAV. *Cell* **40**(1), 9-17.

Walker, B. D., Chakrabarti, S., Moss, B., Paradis, T. J., Flynn, T., Durno, A. G., Blumberg, R. S., Kaplan, J. C., Hirsch, M. S., and Schooley, R. T. (1987). HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature* **328**(6128), 345-8.

Walker, C. M., and Levy, J. A. (1989). A diffusible lymphokine produced by CD8+ T lymphocytes suppresses HIV replication. *Immunology* **66**(4), 628-30.

Walker, C. M., Moody, D. J., Stites, D. P., and Levy, J. A. (1986). CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* **234**(4783), 1563-6.

Walter, P., Gilmore, R., and Blobel, G. (1984). Protein translocation across the endoplasmic reticulum. *Cell* **38**(1), 5-8.

Wang, J. H., Yan, Y. W., Garrett, T. P., Liu, J. H., Rodgers, D. W., Garlick, R. L., Tarr, G. E., Husain, Y., Reinherz, E. L., and Harrison, S. C. (1990). Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. *Nature* **348**(6300), 411-8.

Wang, L. X. (2003). Bioorganic approaches towards HIV vaccine design. *Curr Pharm Des* **9**(22), 1771-87.

Wang, S., Pal, R., Mascola, J. R., Chou, T. H., Mboudjeka, I., Shen, S., Liu, Q., Whitney, S., Keen, T., Nair, B. C., Kalyanaraman, V. S., Markham, P., and Lu, S. (2006). Polyvalent HIV-1 Env vaccine formulations delivered by the DNA priming plus protein boosting approach are effective in generating neutralizing antibodies against primary human immunodeficiency virus type 1 isolates from subtypes A, B, C, D and E. *Virology* **350**(1), 34-47.

Wang, S., York, J., Shu, W., Stoller, M. O., Nunberg, J. H., and Lu, M. (2002). Interhelical interactions in the gp41 core: implications for activation of HIV-1 membrane fusion. *Biochemistry* **41**(23), 7283-92.

Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M., Saag, M. S., Komarova, N. L., Nowak, M. A., Hahn, B. H., Kwong, P. D., and Shaw, G. M. (2003). Antibody neutralization and escape by HIV-1. *Nature* **422**(6929), 307-12.

Weiss, C. D., Barnett, S. W., Cacalano, N., Killeen, N., Littman, D. R., and White, J. M. (1996). Studies of HIV-1 envelope glycoprotein-mediated fusion using a simple fluorescence assay. *AIDS* **10**(3), 241-6.

Weiss, R. A. (1993). How does HIV cause AIDS? *Science* **260**(5112), 1273-9.

Weissenhorn, W., Calder, L. J., Dessen, A., Laue, T., Skehel, J. J., and Wiley, D. C. (1997a). Assembly of a rod-shaped chimera of a trimeric GCN4 zipper and the HIV-1 gp41 ectodomain expressed in Escherichia coli. *Proc Natl Acad Sci U S A* **94**(12), 6065-9.

Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., and Wiley, D. C. (1997b). Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387**(6631), 426-30.

Weissenhorn, W., Wharton, S. A., Calder, L. J., Earl, P. L., Moss, B., Aliprandis, E., Skehel, J. J., and Wiley, D. C. (1996). The ectodomain of HIV-1 env subunit gp41 forms a soluble, alpha-helical, rod-like oligomer in the absence of gp120 and the N-terminal fusion peptide. *Embo J* **15**(7), 1507-14.

Welker, R., Kottler, H., Kalbitzer, H. R., and Krausslich, H. G. (1996). Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. *Virology* **219**(1), 228-36.

Whitney, J. B., and Ruprecht, R. M. (2004). Live attenuated HIV vaccines: pitfalls and prospects. *Curr Opin Infect Dis* **17**(1), 17-26.

Whittle, H., Morris, J., Todd, J., Corrah, T., Sabally, S., Bangali, J., Ngom, P. T., Rolfe, M., and Wilkins, A. (1994). HIV-2-infected patients survive longer than HIV-1-infected patients. *AIDS* **8**(11), 1617-20.

WHO (2004). AIDS epidemic update.

WHO (2005). AIDS epidemic update.

Wiktor, S. Z., Nkengasong, J. N., Ekpini, E. R., Adjorlolo-Johnson, G. T., Ghys, P. D., Brattegaard, K., Tossou, O., Dondero, T. J., De Cock, K. M., and Greenberg, A. E. (1999). Lack of protection against HIV-1 infection among women with HIV-2 infection. *AIDS* **13**(6), 695-9.

Wild, C., Greenwell, T., and Matthews, T. (1993). A synthetic peptide from HIV-1 gp41 is a potent inhibitor of virus-mediated cell-cell fusion. *AIDS Res Hum Retroviruses* **9**(11), 1051-3.

Wild, C., Oas, T., McDanal, C., Bolognesi, D., and Matthews, T. (1992). A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. *Proc Natl Acad Sci U S A* **89**(21), 10537-41.

Wilkins, A., Hayes, R., Alonso, P., Baldeh, S., Berry, N., Cham, K., Hughes, A., Jaiteh, K., Oelman, B., Tedder, R., and et al. (1991). Risk factors for HIV-2 infection in The Gambia. *AIDS* **5**(9), 1127-32.

Wilkins, A., Ricard, D., Todd, J., Whittle, H., Dias, F., and Paulo Da Silva, A. (1993). The epidemiology of HIV infection in a rural area of Guinea-Bissau. *AIDS* **7**(8), 1119-22.

Willett, B. J., Picard, L., Hosie, M. J., Turner, J. D., Adema, K., and Clapham, P. R. (1997). Shared usage of the chemokine receptor CXCR4 by the feline and human immunodeficiency viruses. *J Virol* **71**(9), 6407-15.

Willey, R. L., Rutledge, R. A., Dias, S., Folks, T., Theodore, T., Buckler, C. E., and Martin, M. A. (1986). Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. *Proc Natl Acad Sci U S A* **83**(14), 5038-42.

Willey, R. L., Smith, D. H., Lasky, L. A., Theodore, T. S., Earl, P. L., Moss, B., Capon, D. J., and Martin, M. A. (1988). In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. *J Virol* **62**(1), 139-47.

Willey, S., Roulet, V., Reeves, J. D., Kergadallan, M. L., Thomas, E., McKnight, A., Jegou, B., and Dejucq-Rainsford, N. (2003). Human Leydig cells are productively infected by some HIV-2 and SIV strains but not by HIV-1. *AIDS* **17**(2), 183-8.

Witvrouw, M., Pannecouque, C., Switzer, W. M., Folks, T. M., De Clercq, E., and Heneine, W. (2004). Susceptibility of HIV-2, SIV and SHIV to various anti-HIV-1 compounds: implications for treatment and postexposure prophylaxis. *Antivir Ther* **9**(1), 57-65.

Witvrouw, M., Pannecouque, C., Van Laethem, K., Desmyter, J., De Clercq, E., and Vandamme, A. M. (1999). Activity of non-nucleoside reverse transcriptase inhibitors against HIV-2 and SIV. *AIDS* **13**(12), 1477-83.

Wolinsky, S. M., Wike, C. M., Korber, B. T., Hutto, C., Parks, W. P., Rosenblum, L. L., Kunstman, K. J., Furtado, M. R., and Munoz, J. L. (1992). Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. *Science* **255**(5048), 1134-7.

Wong, G. H., Krowka, J. F., Stites, D. P., and Goeddel, D. V. (1988). In vitro anti-human immunodeficiency virus activities of tumor necrosis factor-alpha and interferon-gamma. *J Immunol* **140**(1), 120-4.

Worobey, M., Santiago, M. L., Keele, B. F., Ndjango, J. B., Joy, J. B., Labama, B. L., Dhed, A. B., Rambaut, A., Sharp, P. M., Shaw, G. M., and Hahn, B. H. (2004). Origin of AIDS: contaminated polio vaccine theory refuted. *Nature* **428**(6985), 820.

Wrigley, N. G., Brown, E. B., Daniels, R. S., Douglas, A. R., Skehel, J. J., and Wiley, D. C. (1983). Electron microscopy of influenza haemagglutinin-monoclonal antibody complexes. *Virology* **131**(2), 308-14.

Wrin, T., Crawford, L., Sawyer, L., Weber, P., Sheppard, H. W., and Hanson, C. V. (1994). Neutralizing antibody responses to autologous and heterologous isolates of human immunodeficiency virus. *J Acquir Immune Defic Syndr* **7**(3), 211-9.

Wrin, T., Loh, T. P., Vennari, J. C., Schuitemaker, H., and Nunberg, J. H. (1995). Adaptation to persistent growth in the H9 cell line renders a primary isolate of human immunodeficiency virus type 1 sensitive to neutralization by vaccine sera. *J Virol* **69**(1), 39-48.

Wu, H., Kwong, P. D., and Hendrickson, W. A. (1997). Dimeric association and segmental variability in the structure of human CD4. *Nature* **387**(6632), 527-30.

Wu, H., Myszka, D. G., Tendian, S. W., Brouillette, C. G., Sweet, R. W., Chaiken, I. M., and Hendrickson, W. A. (1996). Kinetic and structural analysis of mutant CD4 receptors that are defective in HIV gp120 binding. *Proc Natl Acad Sci U S A* **93**(26), 15030-5.

Wu, X., Conway, J. A., Kim, J., and Kappes, J. C. (1994). Localization of the Vpx packaging signal within the C terminus of the human immunodeficiency virus type 2 Gag precursor protein. *J Virol* **68**(10), 6161-9.

Wu, Z., Kayman, S. C., Honnen, W., Revesz, K., Chen, H., Vijh-Warrier, S., Tilley, S. A., McKeating, J., Shotton, C., and Pinter, A. (1995). Characterization of neutralization epitopes in the V2 region of human immunodeficiency virus type 1 gp120: role of glycosylation in the correct folding of the V1/V2 domain. *J Virol* **69**(4), 2271-8.

Wyatt, L. S., Earl, P. L., Liu, J. Y., Smith, J. M., Montefiori, D. C., Robinson, H. L., and Moss, B. (2004). Multiprotein HIV type 1 clade B DNA and MVA vaccines: construction, expression, and immunogenicity in rodents of the MVA component. *AIDS Res Hum Retroviruses* **20**(6), 645-53.

Wyatt, R., Desjardin, E., Olshevsky, U., Nixon, C., Binley, J., Olshevsky, V., and Sodroski, J. (1997). Analysis of the interaction of the human immunodeficiency virus type 1 gp120 envelope glycoprotein with the gp41 transmembrane glycoprotein. *J Virol* **71**(12), 9722-31.

Wyatt, R., Moore, J., Accola, M., Desjardin, E., Robinson, J., and Sodroski, J. (1995). Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. *J Virol* **69**(9), 5723-33.

Wyatt, R., and Sodroski, J. (1998). The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280**(5371), 1884-8.

Xiang, Z., Ariyoshi, K., Wilkins, A., Dias, F., Whittle, H., and Breuer, J. (1997). HIV type 2 pathogenicity is not related to subtype in rural Guinea Bissau. *AIDS Res Hum Retroviruses* **13**(6), 501-5.

Xiao, X., Wu, L., Stantchev, T. S., Feng, Y. R., Ugolini, S., Chen, H., Shen, Z., Riley, J. L., Broder, C. C., Sattentau, Q. J., and Dimitrov, D. S. (1999). Constitutive cell surface association between CD4 and CCR5. *Proc Natl Acad Sci U S A* **96**(13), 7496-501.

Xu, Y. F., Zhang, Y. Q., Xu, X. M., and Song, G. X. (2006). Papillomavirus virus-like particles as vehicles for the delivery of epitopes or genes. *Arch Virol* **151**(11), 2133-48.

Yamaguchi, J., Devare, S. G., and Brennan, C. A. (2000). Identification of a new HIV-2 subtype based on phylogenetic analysis of full-length genomic sequence. *AIDS Res Hum Retroviruses* **16**(9), 925-30.

Yang, X., Farzan, M., Wyatt, R., and Sodroski, J. (2000a). Characterisation of Stable, Soluble Trimers Containing Complete Ectodomains of Human Immunodeficiency Virus Type 1 envelope Glycoproteins. *Journal of Virology* **74**(12), 5716-5725.

Yang, X., Florin, L., Farzan, M., Kolchinsky, P., Kwong, P. D., Sodroski, J., and Wyatt, R. (2000b). Modifications that stabilize human immunodeficiency virus envelope glycoprotein trimers in solution. *J Virol* **74**(10), 4746-54.

Yang, X., Lee, J., Mahony, E., Kwong, P. D., Wyatt, R., and Sodroski, J. (2002). Highly Stable Trimers formed by Human Immunodeficiency Virus Type 1 Envelope Glycoproteins Fused with the Trimeric Motif of T4 Bacteriophage Fibritin. *Journal of Virology* **76**(9), 4634-4642.

Yang, X., Mahony, E., Holm, G. H., Kassa, A., and Sodroski, J. (2003). Role of the gp120 inner domain beta-sandwich in the interaction between the human immunodeficiency virus envelope glycoprotein subunits. *Virology* **313**(1), 117-25.

Yang, X., Wyatt, R., and Sodroski, J. (2001). Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. *J Virol* **75**(3), 1165-71.

Yang, Z., Mueser, T. C., Kaufman, J., Stahl, S. J., and Wingfield, P. T. (1999). The Crystal Structure of the SIV gp41 Ectodomain at 1.47A Resolution. *Journal of Structural Biology* **121**, 131-144.

Yao, Q., Bu, Z., Vzorov, A., Yang, C., and Compans, R. W. (2003). Virus-like particle and DNA-based candidate AIDS vaccines. *Vaccine* **21**(7-8), 638-43.

Yerly, S., Kaiser, L., Race, E., Bru, J. P., Clavel, F., and Perrin, L. (1999). Transmission of antiretroviral-drug-resistant HIV-1 variants. *Lancet* **354**(9180), 729-33.

York, J., and Nunberg, J. H. (2004). Role of hydrophobic residues in the central ectodomain of gp41 in maintaining the association between human immunodeficiency virus type 1 envelope glycoprotein subunits gp120 and gp41. *J Virol* **78**(9), 4921-6.

Yu, X., Matsuda, Z., Yu, Q. C., Lee, T. H., and Essex, M. (1993). Vpx of simian immunodeficiency virus is localized primarily outside the virus core in mature virions. *J Virol* **67**(7), 4386-90.

Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P., and Yu, X. F. (2003). Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* **302**(5647), 1056-60.

Yu, X., Yuan, X., Matsuda, Z., Lee, T. H., and Essex, M. (1992). The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. *J Virol* **66**(8), 4966-71.

Yu, X. F., Wang, Z., Beyrer, C., Celentano, D. D., Khamboonruang, C., Allen, E., and Nelson, K. (1995). Phenotypic and genotypic characteristics of human immunodeficiency virus type 1 from patients with AIDS in northern Thailand. *J Virol* **69**(8), 4649-55.

Yu, X. F., Yu, Q. C., Essex, M., and Lee, T. H. (1991). The vpx gene of simian immunodeficiency virus facilitates efficient viral replication in fresh lymphocytes and macrophage. *J Virol* **65**(9), 5088-91.

Zeh, C., Pieniazek, D., Agwale, S. M., Robbins, K. E., Odama, L., Sani-Gwarzo, N., Gboun, M. S., Inyang, U. S., Folks, T. M., Wambebe, C., and Kalish, M. L. (2005). Nigerian HIV type 2 subtype A and B from heterotypic HIV type 1 and HIV type 2 or monotypic HIV type 2 infections. *AIDS Res Hum Retroviruses* **21**(1), 17-27.

Zekeng, L., Obiang Sima, J., Hampl, H., Ndemesogo, J. M., Ntutumu, J., Sima, V., Devare, S., Kaptue, L., and Gurtler, L. (1997). Update on HIV-1 group O infection in Equatorial Guinea, Central Africa. *AIDS* **11**(11), 1410-2.

Zekeng, L., Salla, R., Kaptue, L., Pinay, P., Barth, P., Schmidt-Ehry, B., and Rehle, T. (1992). HIV-2 infection in Cameroon: no evidence of indigenous cases. *J Acquir Immune Defic Syndr* **5**(3), 319-20.

Zhang, C. W., Chishti, Y., Hussey, R. E., and Reinherz, E. L. (2001a). Expression, purification, and characterization of recombinant HIV gp140. The gp41 ectodomain of HIV or simian immunodeficiency virus is sufficient to maintain the retroviral envelope glycoprotein as a trimer. *J Biol Chem* **276**(43), 39577-85.

Zhang, H., Yang, B., Pomerantz, R. J., Zhang, C., Arunachalam, S. C., and Gao, L. (2003). The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* **424**(6944), 94-8.

Zhang, Y. J., Jin, N. Y., Jiang, W. Z., Zhu, X. J., and Shen, J. C. (2001b). Cloning and expression of the external-glycoprotein gene mutant from HIV-2 in the methylotrophic yeast Pichia pastoris and identification of the glycoprotein. *Biotechnol Appl Biochem* **34**(Pt 1), 1-4.

Zhang, Y. J., Jin, N. Y., Jin, H. T., and Shen, J. C. (2002). Purification and reactiongenicity of human immunodeficiency virus type 2 total external glycoprotein expressed in Pichia Pastoris. *Journal of Virological Methods* **104**(2), 167-172.

Zheng, Y. H., Plemenitas, A., Fielding, C. J., and Peterlin, B. M. (2003). Nef increases the synthesis of and transports cholesterol to lipid rafts and HIV-1 progeny virions. *Proc Natl Acad Sci U S A* **100**(14), 8460-5.

Zhu, P., Chertova, E., Bess, J., Jr., Lifson, J. D., Arthur, L. O., Liu, J., Taylor, K. A., and Roux, K. H. (2003). Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. *Proc Natl Acad Sci U S A* **100**(26), 15812-7.

Zhu, P., Olson, W. C., and Roux, K. H. (2001). Structural flexibility and functional valence of CD4-IgG2 (PRO 542): potential for cross-linking human immunodeficiency virus type 1 envelope spikes. *J Virol* **75**(14), 6682-6.

Zhu, T., Korber, B. T., Nahmias, A. J., Hooper, E., Sharp, P. M., and Ho, D. D. (1998). An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. *Nature* **391**(6667), 594-7.

Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I., and Littman, D. R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* **393**(6685), 595-9.

Zuber, M., Samuel, K. P., Lautenberger, J. A., Kanki, P. J., and Papas, T. S. (1990). Bacterially produced HIV-2 env polypeptides specific for distinguishing HIV-2 from HIV-1 infections. *AIDS Res Hum Retroviruses* **6**(4), 525-34.